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LOYOLA UNIVERSITY CHICAGO

ESTROGEN REGULATION
OF MIR-181A STABILITY
IN NEURONS

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
MASTERS OF SCIENCE

PROGRAM IN NEUROSCIENCE

BY
CHUN KIM
CHICAGO, ILLINOIS
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LIST OF ABBREVIATIONS

ADAR	Adenosine deaminase acting on RNA
AGO	Argonaute protein
cAMP	Cyclic adenosine monophosphate
CDK	Cyclin dependent kinases
DBR1	Lariat debranching enzyme
DCP1a	Decapping enzyme 1a
DCS-1	Decapping scavenger protein
DGCR8	DiGeorge Syndrome Critical Region 8
DRB	5,6-dichlorobenzimidazole 1- β -D-ribofuranoside
dsRBD	dsRNA binding domain
E2	17 β -estradiol
EGTA	Ethylene glycol tetraacetic acid
ERK	Extracellular-signal-regulated kinases
ERE	Estrogen response element
GP1R	G Protein-Coupled Estrogen Receptor 1
GR	Glucocorticoid receptor
Grb2	Growth factor receptor-bound protein 2
KSRP	KH-type splicing regulatory protein
MAPK	Mitogen activated protein kinase
MNase	Micrococcal nuclease
PAPD4	Poly-A polymerase associated domain 4
PNRC2	Proline rich nuclear receptor coactivator 2
PRKRA	Protein kinase interferon-inducible double stranded RNA dependent activator
RIIIDs	RNase III domains
RISC	RNA induced silencing complex

RRP6	Ribosomal RNA processing protein 6
RT PCR	Real time polymerase chain reaction
SCRM	Scrambled siRNA
SDNs	Small RNA degrading nucleases
Shc	Src-homology and collagen homology
TGF- β	Transforming growth factor β
TRBP	TAR RNA-binding protein
TUT4	Terminal uridylyltransferase 4
XRN1	Exoribonuclease 1

CHAPTER ONE

OVERVIEW AND HYPOTHESES

Micro-RNAs, or miRNAs, are a class of non-coding RNAs that are single stranded and act to post-transcriptionally regulate mRNA expression. The biogenesis pathway of miRNAs and their role in suppressing translation through mRNA cleavage have been well described; however, it is still unclear what cellular signaling mechanisms regulate the degradation of mature miRNA. Recently, E2, the primary circulating estrogen, has been shown, *in vivo*, to differentially regulate neuronal miRNA expression in a brain region-specific manner (Rao *et al.*, 2013, *Endocrinology*). Specifically, miR-181a was significantly increased upon estrogen treatment in the ventral and dorsal hippocampus; however, miR-181a precursor and primary transcript levels remained unchanged, suggesting that estrogen is not upregulating miR-181a transcription, but rather, stabilizing the mature form (Rao *et al.*, 2015, *Oncotarget*). This led to the hypothesis that estrogen stabilizes miR-181a in neurons by altering the pool of endogenous RNA and by recruiting coregulatory proteins away from the degradation machinery.

The following specific aims were developed to test the proposed hypothesis.

1) Evaluate the dependence of estrogen-mediated stabilization of miR-181a on the presence of endogenous RNA.

miRNAs can be stabilized upon base pairing to endogenous mRNA targets (Rüegger *et al.*, 2012, *Biochemical Sciences*). Based on the understanding that estrogen activates a variety of intracellular signaling pathways affecting transcription, I predicted that the estrogen-mediated stabilization of miR-181a will be abolished upon degrading endogenous RNA with micrococcal nuclease.

2) Identify the role of the coregulatory protein PNRC2 in miRNA degradation.

PNRC2 (proline-rich nuclear receptor coregulatory protein 2) has been shown to interact with estrogen receptors (ERs) in a ligand-dependent manner to promote transcription (Zhou *et al.*, 2006, *Nucleic Acids Research*). However, PNRC2 also has a distinct role in mRNA degradation; it binds to and activates mRNA-decapping enzymes such as DCP1 (Lai *et al.*, 2012, *Structure*). Interestingly, in *Caenorhabditis elegans*, the decapping scavenger enzyme DCS-1, an analog of DCP1, was found to be crucial for exoribonuclease 1 (XRN-1) activity in the degradation of miRNAs (Meziane *et al.*, 2013, *Cell Cycle*). Therefore, I predicted that upon estrogen treatment, there would be increased association of PNRC with ERs, and a corresponding decrease in the association of PNRC and DCP1a, which subsequently leads to a decrease in XRN-1 recruitment and miRNA stabilization.

CHAPTER TWO

LITERATURE REVIEW

ESTROGEN SIGNALING PATHWAY

Estrogens are a class of steroid hormones that are essential for various cellular processes such as proliferation, metabolism, and growth. 17 β -estradiol (E2), the predominant form of estrogen in the human body, is synthesized from cholesterol after multiple enzymatic processing events. E2 can initiate cellular signaling cascades by interacting with estrogen receptors, ER α and ER β . The binding of E2 to the receptor results in conformational changes that allow for ER α and ER β to dimerize. The dimerized receptors are then translocated to the nucleus where they bind to estrogen response elements (ERE) to facilitate gene expression (Marino *et al.*, 2006, *Curr. Gen.*). Both homodimers and heterodimers of ER α and ER β can be formed upon ligand binding, and various combinations of the dimer have been shown to differentially regulate transcription. There are also multiple isoforms of ER α and ER β resulting from splice variants, and these isoforms further contribute to the complexity and diversity of estrogen mediated gene transcription (Kampa *et al.*, 2013, *Hormones*; Shults *et al.*, 2015, *Endocrinology*).

E2 can also trigger signaling cascades that have non-genomic effects.

GP130, a G-protein coupled receptor located in the endoplasmic reticulum, has also been identified as a receptor for E2. Ligand binding dissociates the tripartite G protein subunits in the intraluminal space of the endoplasmic reticulum. This results in the activation of adenylyl cyclase activity, and cyclic adenosine monophosphate (cAMP) levels are consequently increased. G protein activation can also lead to intracellular calcium mobilization, activation of a variety of kinase cascades, and regulation of monovalent ion channels (Prossnitz *et al.*, 2008, *Physiology*). In the context of the brain, these signaling pathways initiated by E2 can ultimately lead to neuroprotection, neurogenesis, and increased neurotransmission (Arevalo *et al.*, 2015, *Nat Rev Neurosci*).

The complexity of E2 signaling is particularly important for human health as it is known to cause neuroprotective and cardioprotective effects in pre-menopausal women. However, following the menopausal transition and loss of circulating 17 β -estradiol, there are differential effects of this hormone in various tissues. Estrogen replacement therapy can cause proliferation of breast and uterine cancer, increase the risk of cardiovascular disease, and lead to neuronal cell loss (Prentice *et al.*, 2006, *Epub*). It remains unknown how the effects of E2 are modified in such diverse and cell-specific ways.

miRNA BIOGENESIS

miRNAs are a subfamily of noncoding RNAs that post-transcriptionally regulate gene expression. The mature miRNA, between 20 and 24 nucleotides in length, binds to complementary sequences in the 3' UTR of target mRNAs to either cause endonucleolytic cleavage or to repress translation. Endonucleolytic

cleavage of the target mRNA is frequently observed in plants whereas translational repression of the target mRNA seems to be the predominant mode of regulation in metazoans (Ameres *et al.*, 2013, *Nature*).

miRNAs are transcribed in the nucleus by RNA polymerase II (RNA Pol II) into long primary miRNAs (pri-miRNA), which typically contain a stem loop region, a 5' cap, and a 3' poly-A tail. Pri-miRNAs are generally transcribed independently through their own promoter interactions with the canonical transcriptional machinery; however, some miRNAs arise from the introns of protein coding genes. Once transcribed, pri-miRNAs are processed by the microprocessor complex which consists of the endonuclease Drosha and a cofactor called DiGeorge Syndrome Critical Region 8 (DGCR8) (Gregory *et al.*, 2004 *Nature*). DGCR8 is critical for pri-miRNA recognition by Drosha (Han *et al.*, 2004 *Genes Dev*). The C-terminus of DGCR8 contains a protein-protein interaction domain which allows it to bind to Drosha and two double-stranded RNA binding domains (dsRBD) which recognize and interact with pri-miRNAs (Denli *et al.*, 2004, *Nature*). Drosha is a nuclear endonuclease specific for double-stranded RNA sequences. It contains two RNase III domains (RIIIDs) that sequentially cleave the 5' lower stem and the 3' overhang of the pri-miRNA. Once the recognition and cleavage of the pri-miRNA is complete, the resulting structure is precursor miRNA, or pre-miRNA. Pre-miRNA is then transported to the cytoplasm by exportin 5 which forms a complex with Ran, a Ras related GTPase (Yi *et al.*, 2003, *Genes Dev*).

Once in the cytoplasm, the pre-miRNA is then cleaved once more by another RNase III enzyme called Dicer. Dicer has an N-terminal helicase domain that interacts with the stem loop of the pre-miRNA to facilitate recognition (Ketting *et al.*, 2001, *Genes Dev*). Similarly, the PAZ (Piwi-Ago-ZWILLE) domain facilitates the recognition and binding of the terminal ends of the pre-miRNA (Macrae *et al.*, 2006, *Science*). Once the precursor is successfully bound, tandem RIIIDs of Dicer cleave the stem loop region producing a miRNA-miRNA* duplex which consists of approximately 45 nucleotides.

This duplex is then loaded onto an argonaute protein (AGO) via an ATP-dependent process, and RISC, or RNA induced silencing complex, is subsequently assembled. Four different AGO proteins (AGO 1-4) exist, but only AGO2 contains an active site on its PIWI domain that can endonucleolytically cleave the target mRNA (Huntzinger *et al.*, 2011, *Nature Rev. Genet.*). Following the loading of the miRNA duplex, unwinding occurs where the passenger strand (miRNA*) is quickly removed and degraded. The resulting single stranded sequence is termed the guide strand. In rare instances, AGO2 can cleave the passenger strand, and the remnants are subsequently removed by the endonuclease C3PO (Liu *et al.*, 2009, *Science*). Typically, however, the duplex is unwound first, and degradation of the passenger strand occurs after its removal.

The selection of the guide strand is determined by a set of specific, yet flexible, criteria. For instance, the strand with the more thermodynamically unstable 5' terminus is generally selected as the guide strand (Khvorova *et al.*, 2003, *Cell*). In addition, there is a preference for uracil to be in the first nucleotide

position when determining the guide strand (Kawamata *et al.*, 2009, *Nature*). However, the criteria for guide strand selection are not necessarily absolute; there are instances where the unfavorable strand is chosen as the guide strand. Since the passenger strand is degraded quickly upon its removal, the relative abundance of the passenger strand is significantly lower compared to the guide strand. The lower relative abundance, however, does not exclude the passenger strand of its mRNA silencing function. While comparatively less potent in its silencing capabilities, the passenger strand can still be involved in mRNA regulation (Chiang *et al.*, 2010, *Genes Dev*).

Once the guide strand is determined, RISC facilitates the interaction of a seed sequence on the 5' end of the guide strand to its complementary mRNA sequence. The seed sequence is only about 6 to 8 nucleotides in length and can therefore base pair with hundreds of complementary mRNA targets. While complementary base pairing can occur along all regions of the mRNA target, silencing activity is mostly observed when the seed sequence binds to the target 3' UTR (Bartel *et al.*, 2009, *Cell*).

There are also other non-canonical pathways of miRNA biogenesis. For example, the pri-miRNA cleavage step by the microprocessor complex can be bypassed. mirtrons, or miRNAs located within introns, are formed directly from mRNA splicing. The branched mirtron is then converted into a precursor-like structure by the Lariat debranching enzyme (DBR1) (Babiarz *et al.*, 2008, *Genes Dev*). Likewise, processing of the precursor miRNA by Dicer can also be circumvented. For example, pre-miR-451 is directly loaded onto RISC as its

nucleotide sequence is too short to be recognized by Dicer. Once loaded, pre-miR-451 is trimmed by ribonucleases to facilitate its maturation (Cheloufi *et al.*, 2010, *Nature*).

In addition to these non-canonical pathways, there are still other processes that can introduce variations to miRNA biogenesis and development. For instance, isomirs, or miRNA isoforms, exhibit sequence heterogeneity introduced not only by imprecise cleavage, but also by the addition of nucleotides. Dicer, for example, can cleave the precursor form at varying sites resulting in a diverse array of duplexes with different seed nucleotide arrangements (Berezikov *et al.*, 2011, *Genome Res*). As a consequence, these differences in the 5' end seed sequence can then alter the range of interactions with potential mRNA targets.

The seed sequences can also be changed by the ADAR (adenosine deaminase acting on RNA) enzyme. As its name suggests, ADAR facilitates the conversion of adenosine to inosine. Interestingly, ADAR is primarily expressed in neural tissue suggesting that miRNAs in the brain are more frequently edited compared to other tissues (Hundley *et al.*, 2010, *Trends Biochem.*). In *Drosophila*, mature miRNAs can also be trimmed from the 3' end by an enzyme called Nibbler (Liu *et al.*, 2011, *Curr. Bio.*). Similarly in humans, a Nibbler-like exoribonuclease trims the 3' end of mature miRNAs to alter its sequence (Han *et al.*, 2011, *Curr. Bio.*). The function of 3' end trimming is not entirely understood, since the seed sequence which is important for target binding is located in the 5' end. However, it has been proposed that 3' end trimming could alter the

degradation kinetics of the mature miRNA and thereby the duration of target mRNA repression. Finally, 3' heterogeneity can also arise from the addition of nucleotides to the 3' end by TNTases, or terminal nucleotidyl transferases (Rissland *et al.*, 2007, *Mol. Cell Bio.*). This 3' tailing process, especially uridylation and polyadenylation, seems to be important for miRNA turnover.

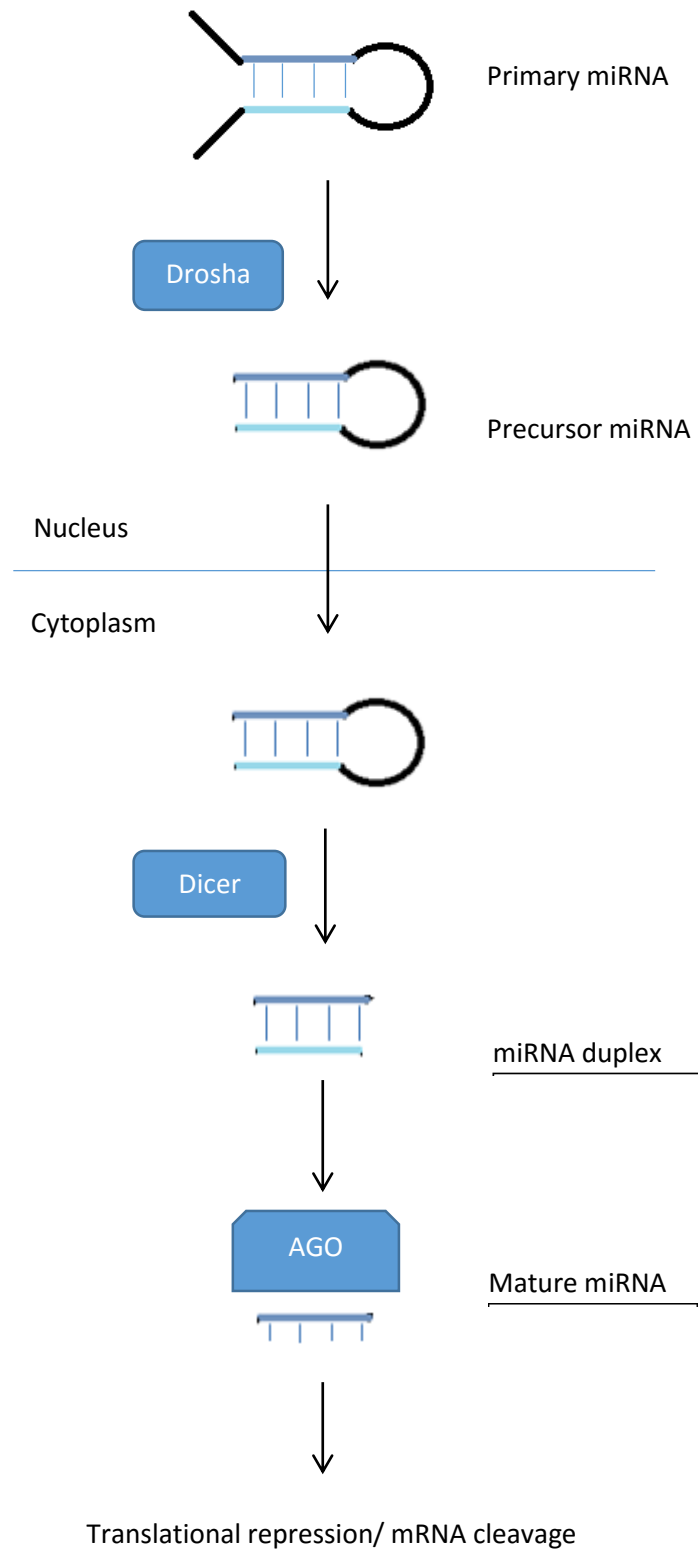


Figure 1. miRNA biogenesis pathway

REGULATION OF miRNA BIOGENESIS

Every step of the miRNA biogenesis pathway can be regulated. As with coding RNA, transcription of miRNA is initiated by various transcription factors interacting with the promotor region. Multiple miRNAs can be transcribed from the same promotor; these miRNAs form a miRNA cluster (Lee *et al.*, 2002, *EMBO*). MYC and p53, transcription factors important for mRNA transcription, can also drive the transcription of miRNA clusters. However, there seems to be specificity of MYC and p53 for particular miRNAs, as binding to some promotor regions induce activation while others induce repression. For example, MYC activates the miR-17 cluster, but generally inhibits the transcription of the miR-15a cluster (Krol *et al.*, 2010, *Nature*). Finally, miRNA transcription can also be regulated by epigenetic means, namely DNA methylation and histone modification (Davis-Dusenbery *et al.*, 2010, *J. Biochem.*).

miRNA biogenesis can also be regulated at the microprocessor step. Since the Drosha:DGCR8 complex is critical for pri-miR cleavage to the precursor form, regulation of this complex can lead to profound changes in miRNA abundance (Han *et al.*, 2009, *Cell*). Interestingly, a form of self-regulation exists between Drosha and DGCR8. DGCR8 stabilizes Drosha allowing for a higher efficiency in the cleavage for Drosha targets. However, *DGCR8* mRNA is one of the many Drosha targets. *DGCR8* mRNA cleavage by Drosha leads to its destabilization, and DGCR8 translation is subsequently diminished. Consequently, less DGCR8 protein is available to bind and stabilize Drosha, and this, in turn, decreases Drosha activity (Yeom *et al.*, 2006, *Nucleic Acids Res.*).

There are also various post-translational modifications of Drosha that can regulate its localization and function. For example, GSK3 β , or glycogen synthase kinase 3 β , phosphorylates Drosha, and this particular modification is required for its nuclear localization (Tang *et al.*, 2010, *Nucleic Acids Res*). Drosha can also be stabilized when acetylated or when bound to TDP43, or TAR DNA-binding protein 43 (Di Carlo *et al.*, 2013, *Mol. Neurobiol.*). Similarly, DGCR8 can also be post-translationally modified. For example, ERK can phosphorylate DGCR8 to promote its stability, and HDAC1, or histone deacetylase 1, can deacetylate DGCR8 to increase its binding affinity to pri-miRNAs (Herbert *et al.*, 2013, *Cell Rep.*). Finally, recent studies have shown that phosphorylated MECP2, or methyl-CpG-binding protein 2, can bind to and sequester DGCR8 inhibiting its association with Drosha (Cheng *et al.*, 2014, *Dev. Cell*).

The efficiency of Drosha processing can also be regulated by RNA-binding proteins such as p68 and p72. Intermediate protein interactions can stimulate Drosha processing such as the SMAD and p53 interaction with p68 (Davis *et al.*, 2008, *Nature*). Other RNA-binding proteins, such as KSRP and HNRNPA1, can also bind to the terminal loop of pri-miRNAs to further facilitate Drosha mediated cleavage (Trabucchi *et al.*, 2009, *Nature*). Dicer processing can also be regulated by RNA-binding proteins. KSRP (KH-type splicing regulatory protein), which is important in Drosha-mediated processing, can also stimulate Dicer activity by binding to the terminal loop region of precursor miRNAs. In the case of let-7 pre-miRNA, LIN28 can compete with KSRP for the terminal loop binding site; therefore, with LIN28 bound, dicing of let-7 pre-miRNA is inhibited

which ultimately leads to a decrease in mature let-7 abundance (Trabucchi *et al.*, 2009, *Nature*).

In *Drosophila*, Dicer interacts with two Loquacious (Loqs) isoforms: Loqs-PA and Loqs-PB. Both Loqs-PA and Loqs-PB interact with Dicer through their dsRBDs and are generally required for the processing of pre-miRNA (Forstemann *et al.*, 2005, *PLoS*). In contrast, human Dicer binds to two known dsRBD proteins: TRBP (TAR RNA-binding protein) and PRKRA (protein kinase interferon-inducible double stranded RNA dependent activator) (Garcia *et al.*, 2007, *Biochimie*). TRBP, in turn, can be stabilized by ERK phosphorylation which then can improve the duration and efficiency of Dicer processing (Paroo *et al.*, 2009, *Cell*).

Finally, miRNA biogenesis can also be regulated by post-translationally modifying AGO proteins at the level of RISC assembly. C-P4H(I), or type I collagen prolyl-hydroxylase, is thought to stabilize AGO2 through hydroxylation (Wu *et al.*, 2011, *Mol. Cell. Biol.*). MAPK phosphorylation of AGO2 at Serine 387 facilitates the localization of AGO2 to processing bodies where translational repression of mRNA targets is thought to occur (Zeng *et al.*, 2008, *Biochem.*). However, when AGO2 is phosphorylated at Tyrosine 529, its ability to bind miRNAs is significantly reduced (Rudel *et al.*, 2011, *Nucleic Acids Res*). Therefore, depending on the site of phosphorylation, AGO function can be completely altered. AGO proteins can also be ubiquitinated by E3 ubiquitin ligases to be targeted to the proteasome for degradation (Rybak *et al.*, 2009, *Nature*). The degradation of AGO proteins via the proteasome pathway could

ultimately affect mature miRNA stability as more miRNAs would be unbound and exposed to various exonucleases that are present in the cytoplasm.

miRNAS IN THE BRAIN

Distinct miRNAs have been found uniquely in brain tissue. For example, miR-9 has been shown to be an important factor in neuronal differentiation (Smirnova *et al.*, 2005, *Eur J Neurosci*). Interestingly, miR-9 triggered the differentiation of embryonic stem cells as well as neural stem cells into neurons (Saunders *et al.*, 2010, *Aging*). miR-7, on the other hand, was found to be important for oligodendrocyte development and promoted myelination, while miR-128 has been shown to be essential for synaptogenesis (Adlakha and Saini, 2014, *Molecular Cancer*).

miRNA dysregulation in the brain has also been linked to various neurological disorders. Interestingly, a mutation in the DGCR8 enzyme was found to significantly increase the risk for schizophrenia, suggesting that the insufficient processing of pri-miRNAs was related to the diseased state (Stark *et al.*, 2008, *Nature Genetics*). Postmortem studies of brains from patients with schizophrenia revealed a general decrease in miRNA expression, especially in the prefrontal cortex which is critical for cognition and social behavior (Perkins *et al.*, 2007, *Genome Biology*).

In patients with Alzheimer's disease, miRNAs targeting amyloid precursor protein (APP) such as miR-106a and miR-106b are generally dysregulated (Herbet *et al.*, 2008, *Acad Sci*). Additionally, miR-9 levels decreased drastically when primary neuron cultures were treated with β -amyloid, suggesting that

plaque formation inhibited neurogenesis and regeneration (Schonrock *et al.*, 2010, *PLOS*). In postmortem studies, miR-9 has been found to be consistently downregulated in Alzheimer's patient brains, whereas miR-125b is upregulated (Cogswell *et al.*, 2008, *Journal of AD*). While further research is needed to fully understand the mechanism of how the dysregulation of these specific miRNAs can lead to disease, their pattern of dysregulation offers promising potential as biomarkers for various disease states.

ESTROGEN REGULATION OF miRNA

Interestingly, estrogen has been shown to regulate miRNA expression *in vivo*. However, most of these studies have focused on E2's effect on miRNA in the context of breast cancer. In MCF-7 cells, a human breast cancer cell line, estrogen decreased the expression of miR-206 (Adams *et al.*, 2007, *Mol Endocrinology*). Another study utilized microarrays to compare miRNA expression in MCF-7 cells following estrogen treatment and found up to 38 miRNAs were differentially regulated by estrogen treatment (Pan *et al.*, 2008, *J Cell Mol*). Generally, widespread decreases in miRNA expression levels have been reported upon estrogen treatment in various breast cancer cell lines (Maillot *et al.*, 2009, *Endocrinology*).

However, there have been several miRNAs that have been identified to be upregulated with E2 treatment. Specifically, Bhat-Nakshatri and colleagues found that miR-let-7f and miR-98 showed robust increases of up to a 3-fold difference following estrogen treatment. Interestingly, previous studies showed that E2 also mediated the increased transcription of MYC and E2F2 mRNA, which are

putative mRNA targets for miR-let-7f and miR-98. Knockdown of miR-let-7f and miR-98 confirmed this target interaction, supporting the notion that miR-let-7f and miR-98 act to counterbalance E2-mediated increases in c-Myc and E2F2 to maintain homeostasis (Bhat-Nakshatri *et al.*, 2009, *Nucleic Acids Research*).

Additionally, Chromatin Immunoprecipitation (ChIP) assays were used to determine that miR-21 had a regulatory region responsive to ER α , suggesting that E2 can directly upregulate pri-miR-21 transcription. Interestingly, it has also been shown that Dicer mRNA can be upregulated through ER α -mediated transcription, potentially introducing an additional layer of complexity involving pre-miRNA processing leading, ultimately, to alterations in miRNA abundance (Bhat-Nakshatri *et al.*, 2009, *Nucleic Acids Research*). Furthermore, miR-222, miR-221, and miR-29a levels were significantly increased in ER α negative breast cancer cells. This finding was interesting, because these miRNAs were shown to repress Dicer levels by binding to the 3' UTR of *Dicer1* mRNA. These results taken together provide even more clarity as to why ER α negative breast cancer cells express low levels of Dicer (Cochrane *et al.*, 2010, *Horm Cancer*).

Furthermore, some specific miRNAs, such as miR-22, have been shown to directly target the 3' UTR of ER α mRNA to inhibit its translation. Consequently, the overexpression of miR-22 resulted in the inhibition of ER α levels and thereby reduced ER α mediated estrogen signaling pathways (Pandey *et al.*, 2009, *Molecular and Cellular Biology*). Therefore, it can be reasonably inferred that the estrogen signaling pathway and the miRNA biogenesis pathway seem to be

intricately intertwined at various intersections—each pathway having the ability to regulate the other.

Unfortunately, there have been few studies that have investigated estrogen regulation of miRNA expression in a context removed from breast cancer research. Recently, estrogen has been shown, *in vivo*, to differentially regulate neuronal miRNA expression in a brain region-specific manner (Rao *et al.*, 2013, *Endocrinology*). In this study, microarray analysis revealed that 34 miRNAs were significantly regulated by estrogen treatment in the ventral hippocampus. Interestingly, E2 had no effect on the processing steps of miRNA biogenesis. The steady state levels of Drosha, Dicer, and AGO2 were not altered with estrogen treatment, suggesting that changes in miRNA levels could be due to E2 acting directly at the promotor level by interacting with estrogen receptors to potentiate pri-miRNA transcription. The other intriguing possibility for the changes in miRNA expression levels could be further downstream—where the stability of mature miRNAs is regulated by E2.

miRNA DEGRADATION

Recent research on miRNA stability has primarily involved identifying key enzymes that are part of the degradation process in both plants and animals. Certain candidate nucleases, such as small RNA degrading nucleases (SDNs) have been proposed to degrade miRNAs in *Arabidopsis thaliana* from the 3'-to-5' direction (Ramachandran *et al.*, 2008, *Science*). In *Caenorhabditis elegans* and in humans, the 5'-to-3' exoribonuclease XRN-1, is critical for miRNA degradation (Chatterjee *et al.*, 2011, *Dev. Cell*). Recently, it has been revealed that a critical

enzyme in miRNA degradation is the decapping scavenger protein DCS-1. This finding was interesting due to the fact that mature miRNAs, unlike mRNAs, do not have a 5' cap. DCS-1 was important for another reason—it recruited XRN1, an exoribonuclease, to the miRNA to initiate 5' to 3' degradation (Bosse *et al.*, 2013, *Molecular Cell*). Therefore, the vast array of unique enzymes found to be important for miRNA degradation seem to oppose the notion of a general, widely conserved pathway for miRNA degradation; rather, different organisms seem to rely on distinct mechanisms of degradation.

Generally, it has been observed that miRNAs are globally more stable than mRNA (Gantier *et al.*, 2011, *Nucleic Acids Res*). In addition, highly expressed miRNAs are correlated with a higher rate of turnover whereas lowly expressed miRNAs seem to be relatively more stable. miRNA turnover kinetics also varied with cell type, affirming the hypothesis that miRNA stability is context dependent (Li *et al.*, 2013, *BioMed Research Int.*).

The specific triggers that regulate the miRNA degradation process is, however, still unclear. One intriguing possibility is that miRNA turnover is mediated by its mRNA target. While mRNAs are generally thought to be translationally repressed or cleaved by interacting with mature miRNAs loaded onto RISC, there have been studies that suggest a reciprocal means of regulation where miRNA stability can be influenced by mRNA as well. For instance, in HEK293T cells, miRNAs were degraded more rapidly upon base pairing with highly complementary mRNA targets compared to pairing with

mRNA targets with less than 8 nucleotide lengths of complementarity (Ameres *et al.*, 2010, *Science*).

Cell cycle progression has also been observed as a regulator of miRNA stability. Specific cellular environments are thought to accelerate miRNA degradation, especially during cell cycle transitional phases. A proposed mechanism of miRNA degradation driving cell cycle progression closely mirrors the rapid induction and turnover of various cyclins which activate CDKs, or cyclin dependent kinases, to initiate a signaling cascade specific to the phase of the cell cycle. For instance, miR-29b was found to be enriched in mitotic cells. The half-life of miR-29b was greater than 12 hours in mitotic cells compared to approximately 4 hours in non-dividing cells (Hwang *et al.*, 2007, *Science*). Some miRNAs are expressed constitutively at almost undetectable levels so that its activity can be quickly abolished when necessary. An example of this is miR-503 which was observed in growth arrested cells but became undetectable upon re-entry to the cell cycle (Rissland *et al.*, 2011, *Mol. Cell*). This finding was intuitive, as the factors important for cell cycle progression, cyclin D1, cyclin E1, and CDK6, were all found to be putative targets for miR-503.

Recently, there have been some interesting studies investigating the possibility of miRNA turnover regulated by extracellular signals. For example, EGF (epidermal growth factor) treatment rapidly decreased levels of 23 miRNAs in MCF10A breast epithelial cells (Avraham *et al.*, 2010, *Sci. Signal*). Common targets of these miRNAs included immediate early genes and other growth promoting genes. These findings suggest that EGF-mediated proliferation is

activated partly by the rapid turnover of several growth-inhibiting miRNAs. These studies, unfortunately, failed to differentiate between miRNA degradation and repression of their expression.

Rapid turnover, however, seems to be a prevailing feature of miRNAs in neurons. One study examined miRNA turnover in embryonic stem cells in relation to differentiated pyramidal neurons and found rapid miRNA turnover in pyramidal neurons but not in the undifferentiated state (Krol *et al.*, 2010, *Cell*). miRNAs taken from primary human neuronal cultures and from post-mortem brain tissues also exhibited significantly shorter half-lives of less than three and a half hours, as compared to those reported in other non-differentiated cells (Sethi *et al.*, 2009, *Neuroscience Letters*).

This rapid turnover in neurons seems to be dependent on neuronal activity. When tetrodotoxin was used to block action potential formation, the rapid degradation of most miRNAs was prevented. The major exception to this rule was miR-132 which experienced rapid decay when glutamate receptors were experimentally blocked (Krol *et al.*, 2010, *Cell*). Otherwise, a positive correlation between neuronal activity and rapid miRNA decay was observed. Another study reported that XRN-2 and PAPD4—an exoribonuclease and a poly-A polymerase important for miRNA degradation—were expressed weakly in immature neurons, but levels seemed to accumulate as maturation occurred. Interestingly, XRN-2 and PAPD4 levels were virtually nonexistent in glial and endothelial cells, supporting the notion that neurons are distinct in their characteristic of having higher rates of miRNA degradation (Kinjo *et al.*, 2013, *Exp. Neurology*).

Post-transcriptional modifications to the miRNA are another way stability can be regulated. In HEK293 cells, knockdown of the m6A demethylase, FTO, resulted in the steady state level change of 17 miRNAs, implying that methylation of adenosine could be important in determining the stability of a subset of miRNAs (Berulava *et al.*, 2015, *PLOS*). In plants, the 3' end of miRNAs is methylated by the methyltransferase HEN1. This methylation offers effective, albeit incomplete, protection from being degraded by small RNA degrading nucleases (SDNs) (Li *et al.*, 2005, *Curr. Biol.*).

Oligouridylation, where 10 to 30 nucleotides are added to the 3' end of the miRNA by the terminal nucleotidyl transferase HESO1, accelerated miRNA degradation by SDNs, suggesting that uridylation either promoted the assembly of the degradation machinery or provided the fully formed machinery with a more preferable docking site (Zhao *et al.*, 2012, *RNA Biol*). A similar effect of instability mediated by uridylation can be seen in *Chlamydomonas reinhardtii* where a different terminal nucleotidyl transferase called MUT68 is responsible for uridylating the 3' ends of miRNAs. Unsurprisingly, this uridylation promoted degradation of the miRNA by RRP6, or ribosomal RNA processing protein 6 (Ibrahim *et al.*, 2010, *Proc. Natl. Acad. Sci.*). For human miRNAs, however, a different effect was seen upon uridylation. The nucleotidyl transferase TUTase 4 was found to uridylate miR-26a in human lung alveolar epithelial cells. However, uridylation did not affect miR-26 degradation kinetics; rather, there was a reduction in the efficacy of miR-26a's inhibitory effects on its target interleukin-6 (Jones *et al.*, 2009, *Nat Cell Bio*). To add another layer of complexity, precursor

miRNAs were shown to be uridylated as well, and depending on the specific context of uridylation, the effects of uridylation were vastly different.

Oligouridylation of the pre-miRNA by TUT4 inhibited miRNA biogenesis whereas monouridylation seemed to promote miRNA biogenesis. Interestingly, when the 3' end of the precursor was trimmed by a nuclease prior to uridylation by TUT4, the pre-miRNA was more prone to degradation (Kim *et al.*, 2015, *EMBO*).

Therefore, the consequences of uridylation on miRNA stability seem to be heavily dependent on both species and context.

While polyadenylation has not been studied as extensively as uridylation with respect to miRNA turnover, post-transcriptional modifications involving polyadenylation seem to also be context dependent. miR-122, expressed in hepatocytes, was stabilized upon adenylation; however, adenylation of miRNAs by the virally encoded VP55 protein resulted in rapid decay (Katoh *et al.*, 2009, *Genes Dev.*).

Recent research has also illuminated the ability of viruses to employ an interesting method to destabilize host miRNAs. For instance, *Herpesvirus saimiri* can encode several non-coding RNAs that can base pair with miR-27a.

Specifically, HSUR1 (Herpesvirus saimiri U RNA) was found to interact with the seed sequence of miR-27a to promote its degradation (Cazalla *et al.*, 2010, *Science*). This intriguing finding sheds light on how specific miRNAs can be targeted for degradation to elicit a preferred cellular response—namely, the apparent viral inhibition of a host cell's defense system.

These previous studies have revealed various mechanisms by which miRNA stability can be regulated. Both the biogenesis and degradation of many miRNAs can be specifically targeted, and its effects seem to rely heavily on the context of the cellular and physiological environment. As the field of miRNA research moves forward, the mechanisms of regulation and extracellular cues that negate or potentiate miRNA degradation must be better elucidated. The present study investigated the role of E2 in the miRNA degradation process and introduced hormonal triggers that can elicit differential effects on the turnover process of specific miRNAs and the mechanisms that underlie this process.

CHAPTER THREE
THE ROLE OF ENDOGENOUS RNA
IN THE E2-MEDIATED STABILIZATION OF MIR-181A
INTRODUCTION

The role of endogenously expressed RNAs in mediating miRNA stability has come under investigation in recent years. Single stranded, mature miRNAs are generally thought to be stabilized by binding to their mRNA targets (Chatterjee *et al.*, 2011, *Dev Cell*). While the reason behind target-induced stability of miRNAs is not fully known, it has been hypothesized that association with RISC shields the mature miRNA from various exoribonucleases. Since base pairing interactions between miRNA and target mRNA only occur after the mature strand is loaded onto RISC, a case can be made that an abundance of the target mRNA leads to a change in kinetics where the mature miRNA strand is bound and thereby protected by RISC for a longer period of time. However, this hypothesis has yet to be confirmed. Moreover, the factors that facilitate or initiate the unloading process of the mature miRNA strand from RISC are still unknown.

Interestingly, mRNA targets that have a high degree of complementarity to the mature miRNA sequence destabilize the corresponding miRNA. Specifically for miR-223 in HEK293T cells, a significantly faster rate of degradation was observed when the mature form was incubated with a perfectly

complementary sequence as compared to a sequence that had a two nucleotide bulge in the center (Baccarini *et al.*, 2011, *Curr Bio*).

Antagomirs, or synthetically produced miRNA silencing agents, were developed largely based on this notion of complementarity-induced miRNA degradation. Antagomirs are highly complementary oligonucleotide sequences that contain 2'-O-methylations in the sugar-phosphorothioate backbone along with a cholesterol moiety conjugated to the 3' end. These modifications protect the single stranded antagomir itself from endogenous nucleases while still giving it the ability to bind and initiate the degradation of its target miRNA (Krützfeldt *et al.*, 2007, *Nucleic Acids Res*).

This reciprocal modulation of miRNA by target RNA sequences is also observed in neurons; highly complementary mRNA targets were found to trigger the addition of single nucleotides to the 3' end of the mature miRNA which ultimately lead to 3'-to-5' degradation by endogenous exonucleases (M de la Mata *et al.*, 2015, *EMBO Rep*). Therefore, it can be seen from the literature that endogenous mRNA targets and even synthetically produced oligonucleotide sequences can have profound effects on miRNA stability.

These studies aimed to determine if the presence of endogenous RNA was necessary for the estrogen-mediated stabilization of miR-181a. My hypothesis was that estrogen-mediated stabilization of miR-181a will be abolished in the absence of endogenous RNA. I tested my hypothesis by using a modified version of an *in vitro* miRNA turnover assay described by Chatterjee *et al* (2009, *Nature*).

APPROACH

To first confirm that E2 stabilized miR-181a *in vitro*, IVB cells, derived from rat hypothalamus, were treated with DRB to inhibit transcription. Transcription was inhibited to ensure that E2 could not alter mature miR-181a levels by upregulating transcription. After DRB treatment, IVB cells were incubated with E2 or vehicle for 2 hours to determine the effect of treatment on mature miR-181a.

A miRNA degradation assay was performed as described by Chatterjee *et al* to visualize the degradation of a radiolabeled, mature miR-181a transcript through time. Since the mature form of the miRNA was added into the system exogenously, any effect of E2 observed for miR-181a was assumed to be on the mature form. The miRNA degradation assay was then repeated with a radiolabeled, mature miR-124 construct to determine if the stabilizing effect of E2 was miRNA specific.

Finally, to determine the role of endogenous RNA on the E2-mediated stabilization of miR-181a, a modified form of the miRNA degradation assay was used. Briefly, IVB cells were treated with either E2 or vehicle treatment for 48 hours and then lysed with a mild non-denaturing lysis buffer. Lysates were treated with micrococcal nuclease in order to degrade all nucleotide sequences that were present endogenously. There were two different treatment groups: control (vehicle or estradiol treated IVB cells with EGTA only) and MNase (vehicle or estradiol treated IVB cells with MNase *and* EGTA). After performing the miRNA degradation assay, the levels of radiolabeled miR-181a were determined at each time point by using densitometry through ImageJ software.

The fold change of miR-181a levels from each time point compared to T0 was determined, and a Student's t-test was used to discover significant differences in the fold change of miR-181a levels.

RESULTS

The first experiment aimed to determine the effect of E2 on mature miR-181a levels in rat hypothalamic neurons (IVB). The results showed that E2 treatment increased mature miR-181a levels (Fig. 2a). Similarly, E2 treatment increased miR-181a levels in the presence of a transcriptional inhibitor, DRB (Fig. 2b). These data suggest that the observed E2-induced increases in mature miR-181a were not due to increases in the primary miR-181a transcript.

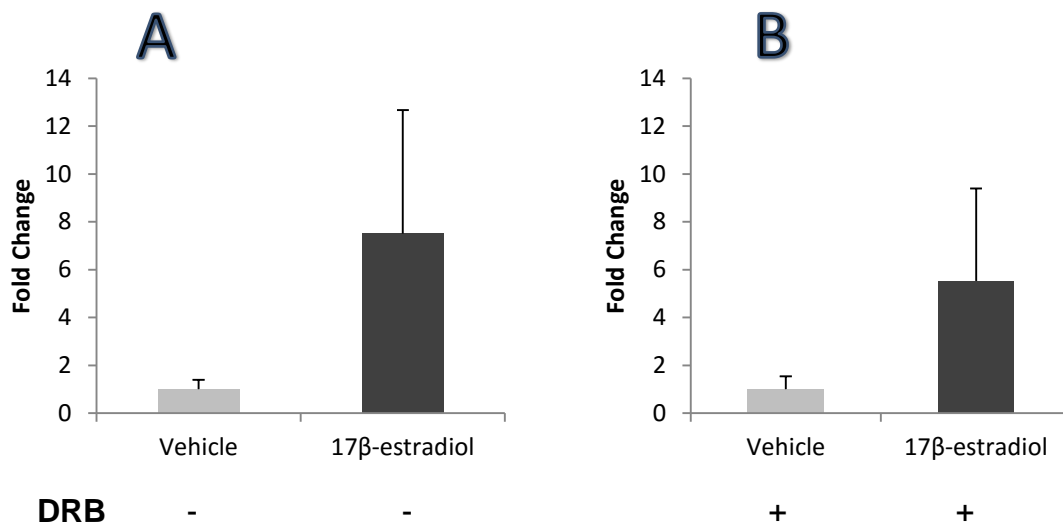


Figure 2. E2 stabilizes miR-181a *in vitro* (A) RT-qPCR results showing relative expression of miR-181a levels between E2-treated and vehicle-treated IVB cells. (B) RT-qPCR results showing fold change in miR-181a levels between E2-treated and vehicle-treated IVB cells following DRB treatment. Two sample t-test was performed to test for significance. * indicates $p < .05$ ($n=3$)

Next, I used a miRNA degradation assay to determine if E2 treatment increased mature miR-181a stability. As shown in Figure 3a, E2 treatment prolonged the levels of mature ^{32}P miR-181a for 120 minutes, whereas the expression was significantly reduced after 60 minutes in vehicle-treated cells. Notably, the appearance of ^{32}P miR-181a degradation products coincided with the disappearance of the mature miRNA (Fig. 3a). Quantitative analysis of 3 independent experiments revealed a statistically significant difference between vehicle and E2 treatment after 120 minutes when the densitometry values were normalized to time zero (T0) (Fig. 3b).

To determine if this stabilizing effect of E2 was specific to miR-181a, I repeated the degradation assay using a radiolabeled nucleotide sequence for miR-124. miR-124 was chosen because it is highly expressed in the rat brain, and is not altered by E2 treatment *in vivo* (Rao *et al.*, 2013, *Endocrinology*). As predicted, the stabilizing effect of E2 treatment was no longer observed in the case of ^{32}P miR-124. ^{32}P miR-124 was present throughout all 5 time points for both treatment groups displaying a longer half-life compared to miR-181a (Fig 4a). Interestingly, E2 treatment even seemed to accelerate ^{32}P miR-124 degradation. At the 240-minute time point, there was a significant decrease in ^{32}P miR-124 radioactivity with E2 treatment, as determined by the densitometry values relative to T0 (Fig. 4b). Therefore, not only was the stabilizing effect of E2 specific to miR-181a, a destabilizing effect was observed when another miRNA was tested.

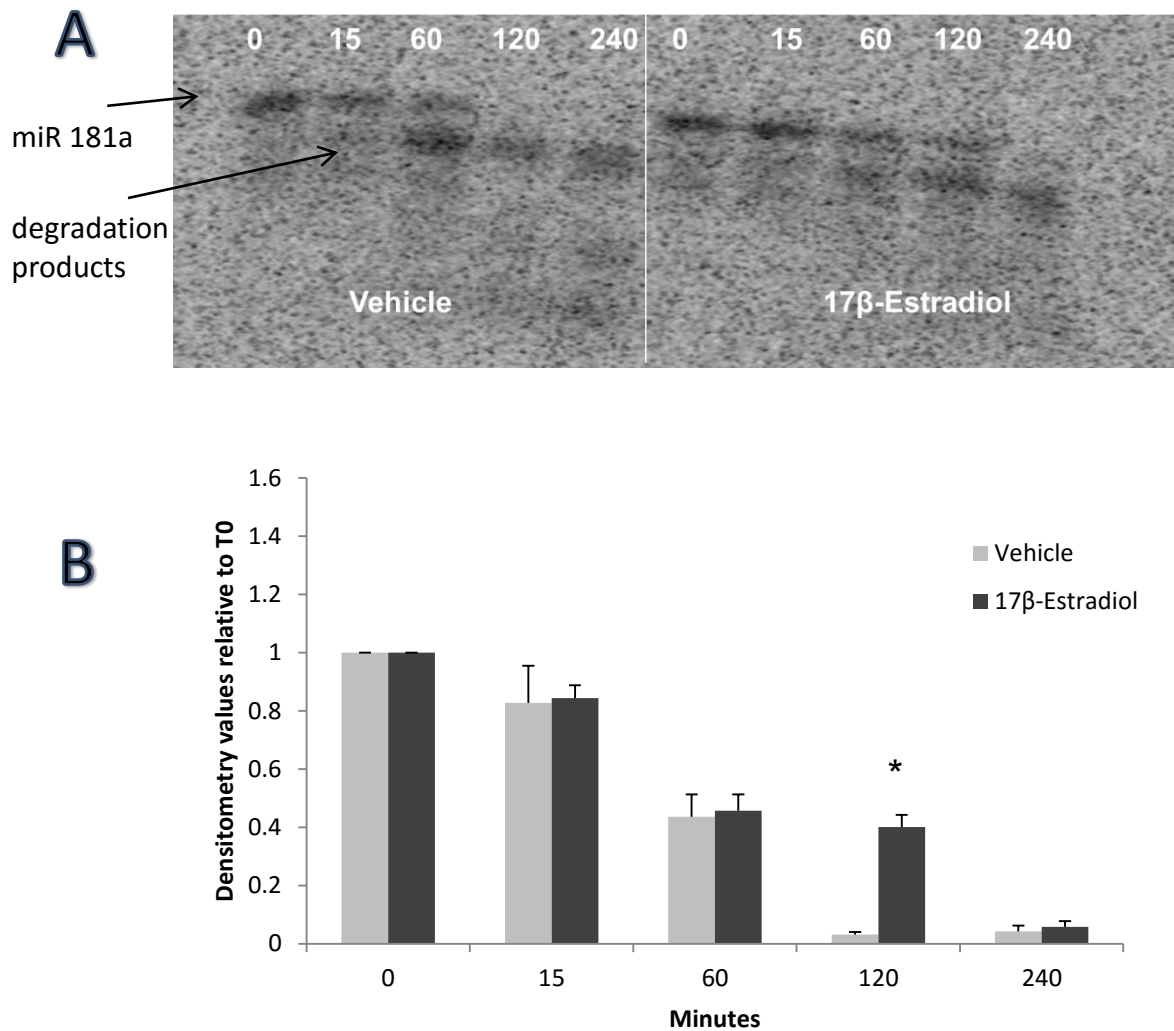


Figure 3. E2-mediated stabilization of miR-181a through time (A) 0, 15, 60, 120, and 240 represent length of time in minutes of radiolabeled miR-181a incubation with lysate. The first 5 time points represent the vehicle treated IVB cells, and the following 5 time points represent the estrogen treated IVB cells. (B) The bar graph depicts densitometry values relative to T0 of radiolabeled miR-181a with E2 or vehicle treatment through time. (N=3) Two sample t-test was performed to examine differences at each time point. * indicates $p < .05$

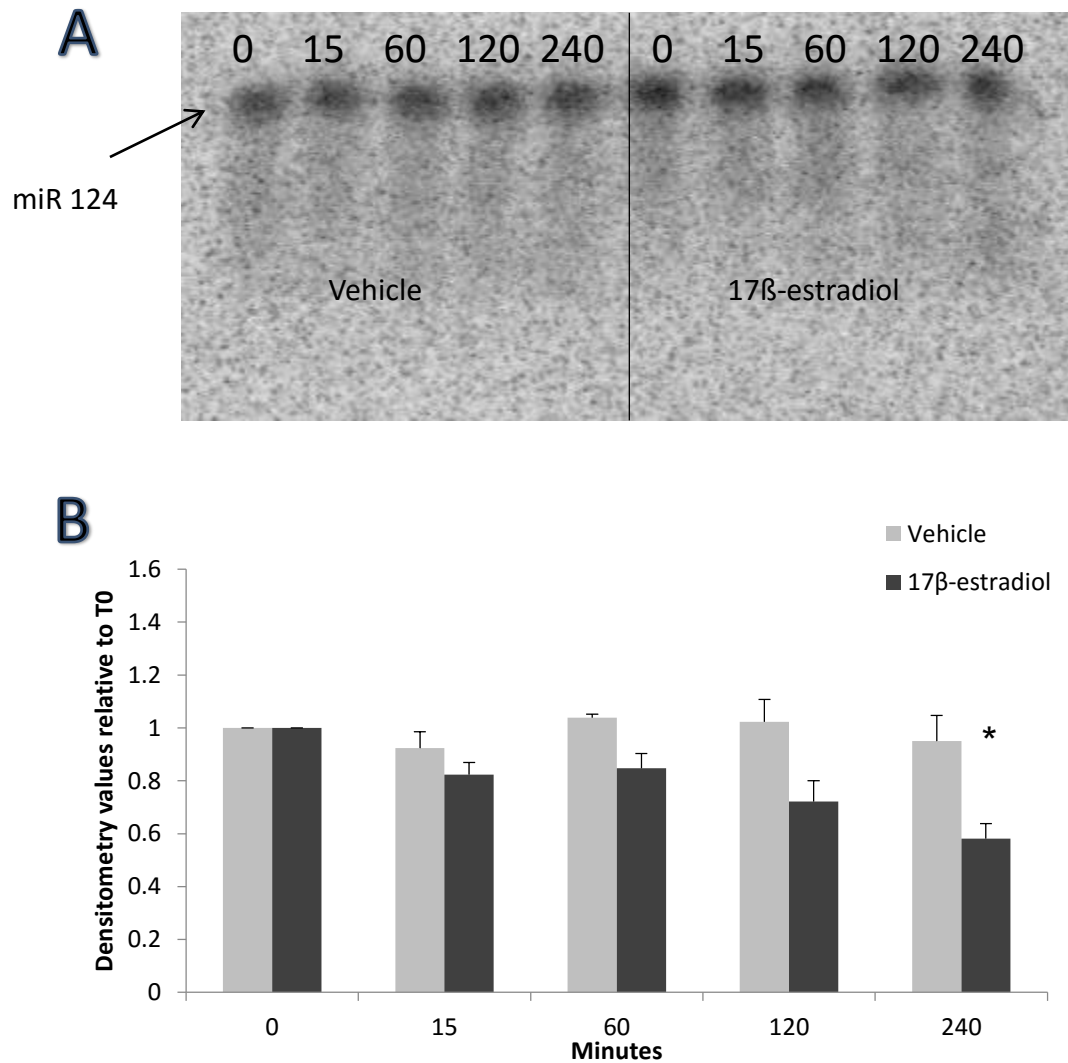


Figure 4. E2-mediated destabilization of miR-124 through time (A) 0, 15, 60, 120, and 240 represent length of time in minutes of radiolabeled miR-124 incubation with lysate. The first 5 time points represent the vehicle treated IVB cells, and the following 5 time points represent the estrogen treated IVB cells. (B) The bar graph depicts densitometry values relative to T0 of radiolabeled miR-124 with E2 or vehicle treatment through time. (N=3) Two sample t-test was performed to examine differences at each time point. * indicates $p < .05$

The next experiment aimed to determine whether the observed E2-mediated stabilization of miR-181a was dependent on the endogenous pool of RNA in the cell lysate. Therefore, cell lysate was first treated with micrococcal nuclease to deplete all endogenous RNA. The nuclease reaction was quenched and ^{32}P miR-181a oligonucleotides were incubated with the lysate according to the previously described miRNA degradation assay protocol. With the endogenous pool of RNA completely depleted from the system, the stabilizing effect of ^{32}P miR-181a was no longer observed at the 120 minute time point (Fig. 5a). Instead, ^{32}P miR-181a expression levels were stable throughout all 5 timepoints independent of vehicle or E2 treatment (Fig. 5b). This suggested that the stabilizing effect of E2 on ^{32}P miR-181a was at least partly mediated by the endogenous pool of RNA since this effect was no longer seen after depleting the system of all RNA.

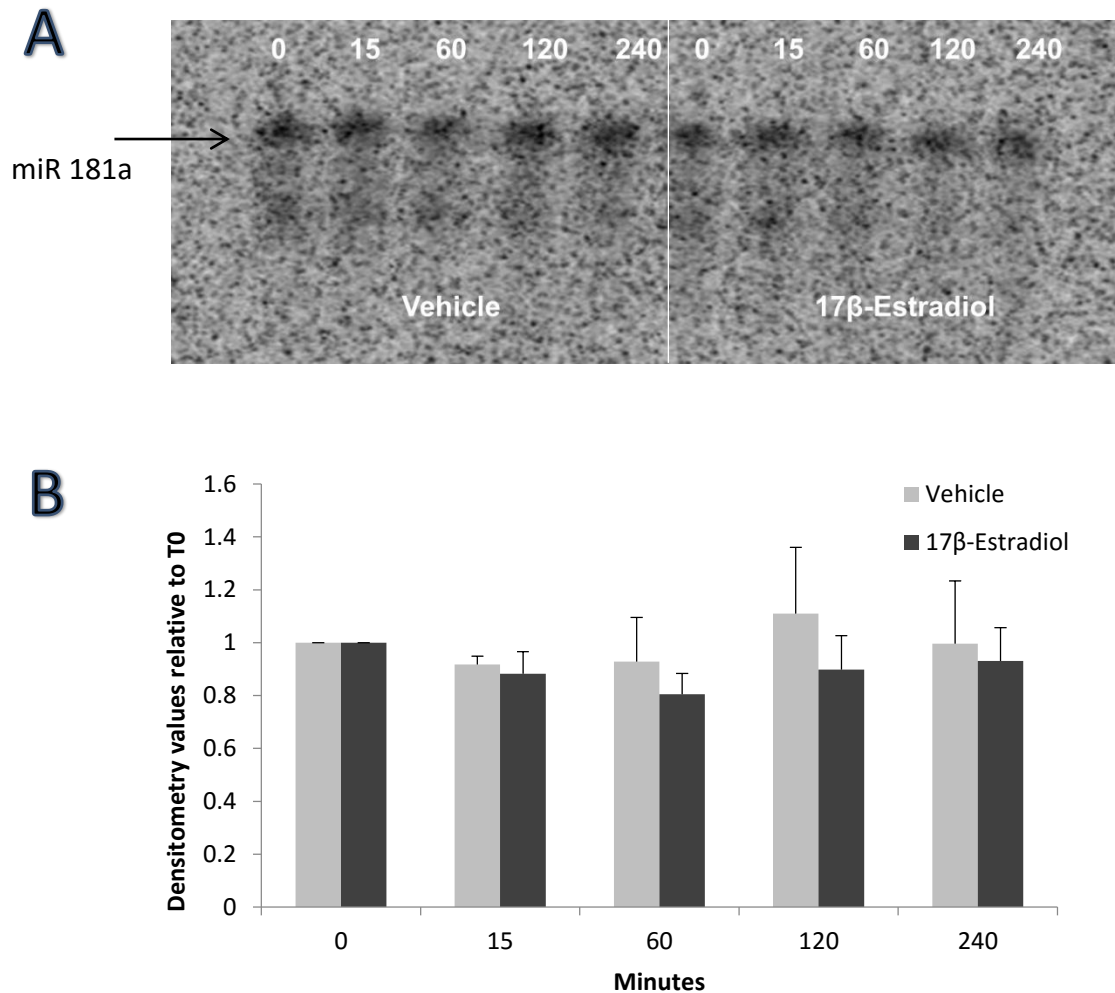


Figure 5. E2-mediated stabilization of miR-181a is abolished with MNase treatment (A) 0, 15, 60, 120, and 240 represent length of time in minutes of radiolabeled miR-181a incubation with lysate. The first 5 time points represent the vehicle treated IVB cells, and the following 5 time points represent the estrogen treated IVB cells.(B) The bar graph depicts densitometry values relative to T0 of radiolabeled miR-181a with E2 or vehicle treatment through time. (N=3) Two sample t-test was performed to examine differences at each time point. * indicates $p < .05$

DISCUSSION

Overall, these studies revealed the novel finding that E2 treatment can differentially affect the degradation kinetics of specific miRNAs in the rat brain. Previous data from our lab suggested that E2 stabilized the mature miR-181a transcript in the rat hypothalamus, since E2 significantly increased mature miR-181a levels with no effect on the primary and precursor forms (Rao *et al.*, 2015, *Oncotarget*). These data suggested that the E2-mediated increase of miR-181a was due to the stabilization of the mature form rather than upregulation of its transcription. This hypothesis was supported by both the miRNA degradation assay using radiolabeled, mature miR-181a (Fig. 3) and the *in vitro* studies utilizing DRB (Fig. 2b).

Interestingly, a different effect of E2 treatment on miR-181a was observed in MCF7 breast tumor cells by Maillot and colleagues. E2 treatment was found to decrease both pri-miR-181a and mature miR-181a expression. Additionally, treatment with the transcriptional inhibitor, actinomycin D, abolished the E2-mediated decrease of miR-181a, suggesting that E2 downregulated the transcription of miR-181a (Maillot *et al.*, 2009, *Cancer Research*). In bone marrow-derived mesenchymal stem cells, miR-181a levels were similarly decreased with E2 treatment (Shao *et al.*, 2015, *FASEB*). Therefore, it seems that E2's effect on the miRNA degradation process is not only miRNA specific, but variable depending on the tissue and cellular environment.

The present findings also indicate that the stabilizing effect of E2 is mediated through the pool of endogenous RNA. These results are consistent with evidence supporting a role for mRNAs in the stabilization of mature miRNAs, although our experimental paradigm precludes ruling out other RNA species (Pasquinelli, 2012, *Nature Reviews Genetics*). Since E2 is a potent initiator of signaling cascades that can lead to alterations in mRNA transcription, it is conceivable that mRNA targets of miR-181a are upregulated resulting in more target-mediated stabilization. This mode of action would explain the miRNA-specific effect of E2 that was observed. While E2 was stabilizing for miR-181a, it destabilized miR-124. Mature miRNA transcripts have a short seed sequence about 2 - 8 nucleotides in length which can provide a binding target for many mRNAs. Therefore, target-mediated stabilization would explain how E2 could have miRNA specific effects.

An alternate possibility is that endogenous RNAs actively destabilize miRNAs, and the micrococcal nuclease degraded some of these destabilizing effectors. However, this interpretation is not supported by evidence in the literature. For example, only virally induced long noncoding RNAs and perfectly complementary mRNA targets have been shown to be destabilizing to their respective miRNAs (Baccarini *et al.*, 2011, *Curr Bio*; Cazalla *et al.*, 2010, *Science*). The IVB cells used in these experiments, to our knowledge, were not virally infected, and perfectly complementary mRNA targets are generally not found in metazoans. However, this possibility cannot be ruled out by my experimental design.

Another interpretation for the stabilization of miR-181a via RNA depletion is that there was increased incorporation of the radiolabeled ^{32}P miR-181a construct into RISC. Since all RNAs were depleted with micrococcal nuclease treatment, the custom radiolabeled ^{32}P miR-181a would not be competing with other endogenous miRNAs for RISC occupancy. If incorporation into RISC is increased, Argonaute-bound miR-181a could be protected from exonucleases by the assembly of proteins that comprise the complex.

The present findings supported my hypothesis that estrogen-mediated stabilization of miR-181a will be abolished in the absence of endogenous RNA. These findings suggest the importance of endogenous RNA in mediating the stabilizing effect of E2 on mature miR-181a levels.

CHAPTER FOUR

THE ROLE OF PNRC2 IN THE E2-MEDIATED STABILIZATION OF MIR-181A

INTRODUCTION

There are multiple ways in which E2 can modulate intracellular signaling processes. In the canonical mechanism of estrogen signaling, E2 binds to estrogen receptors, ER α or ER β . These receptors then dimerize and translocate into the nucleus where they bind to EREs, or estrogen response elements, near the promotor site of target genes. In essence, ERs act as transcription factors to initiate gene expression. Like other transcription factors, ERs can bind to coactivators which further help to facilitate transcription. PNRC2, or proline-rich nuclear receptor coregulatory protein 2, is one family of coactivators that have been shown to bind to estrogen receptors through its SH3-binding motif (Zhou *et al.*, 2006, *Nucleic Acids Res*).

Interestingly, PNRC2 also has a role in mRNA degradation. The unique proline-rich region of PNRC2 has been shown to interact with the EVH1 domain of Dcp1a (Lai *et al.*, 2012, *Structure*). Dcp1a is a decapping enzyme that removes the 5' cap of mRNAs. After the 5' cap is removed, the decapping machinery can recruit XRN1, which subsequently degrades the mRNA from the 5'-to-3' direction (Braun *et al.*, 2012, *Nature*). Importantly, XRN1 is also

responsible for the degradation miRNAs in *C. elegans* and in humans (Bail *et al.*, 2010, *RNA*).

Therefore, these studies aimed to directly link components of the estrogen signaling pathway to miRNA degradation. I hypothesized that E2 treatment would increase the association of PNRC2 with ERs resulting in a corresponding decrease its association with DCP1a. The dissociation of PNRC2 and DCP1a would subsequently decrease XRN-1 recruitment to mature miRNAs leading to increased stabilization. This hypothesis was tested by using a combination of approaches including immunocytochemistry and siRNA knockdown of PNRC2. The results from these studies would provide a novel and specific mechanism for E2-mediated stabilization of miRNAs.

APPROACH

Using the same *in vitro* E2 or vehicle treatment paradigm, immunocytochemistry was performed to visualize differences in PNRC2 localization in IVB cells. After the cells were fixed, primary antibodies to PNRC2 and DCP1a were applied followed by the addition of secondary antibodies conjugated to two different fluorophores. PNRC2 and DCP1a signals were then detected through a fluorescence microscope. Finally, PNRC2's nuclear and cytoplasmic localization and its co-localization with DCP1a were then determined using IMARIS.

To determine if PNRC2 has a functional role in miR-181a stabilization, PNRC2 siRNA was transfected into IVB cells to knockdown its expression. To ensure that any observed changes were due solely to miR-181a stability, IVB cells were initially treated with DRB to inhibit transcription. Following DRB

treatment, cells were incubated with either E2 or vehicle treatment for 2 hours. After verification of PNRC knockdown using Western blotting techniques, RNA was isolated from the various samples. miR-181a levels were then determined by RT-qPCR, and the relative expression of miR-181a was normalized to 5s-rRNA, which is stably expressed in various regions of the brain.

RESULTS

The aim of the first experiment was to determine whether E2 treatment altered the expression or localization of PNRC2 in IVB cells. I hypothesized that more PNRC2 would localize to the nucleus with E2 treatment, since PNRC2 can function as a coactivator to the estrogen receptor-mediated transcription machinery. However, IMARIS software analysis revealed that E2 treatment did not alter the nuclear or cytoplasmic localization of PNRC2 (Fig. 6). Further, there were no significant differences in the ratio of nuclear to cytoplasmic PNRC2 or in the ratio of nuclear to total PNRC2 between the vehicle treated and E2 treated IVB cells (Fig. 7). These effects were not dependent on changes in total PNRC2 content, as E2 treatment did not alter the levels of PNRC2. More importantly, the expression levels of total nuclear and cytoplasmic PNRC2 proteins remained unchanged between cells after E2 or vehicle treatment (Fig. 8).

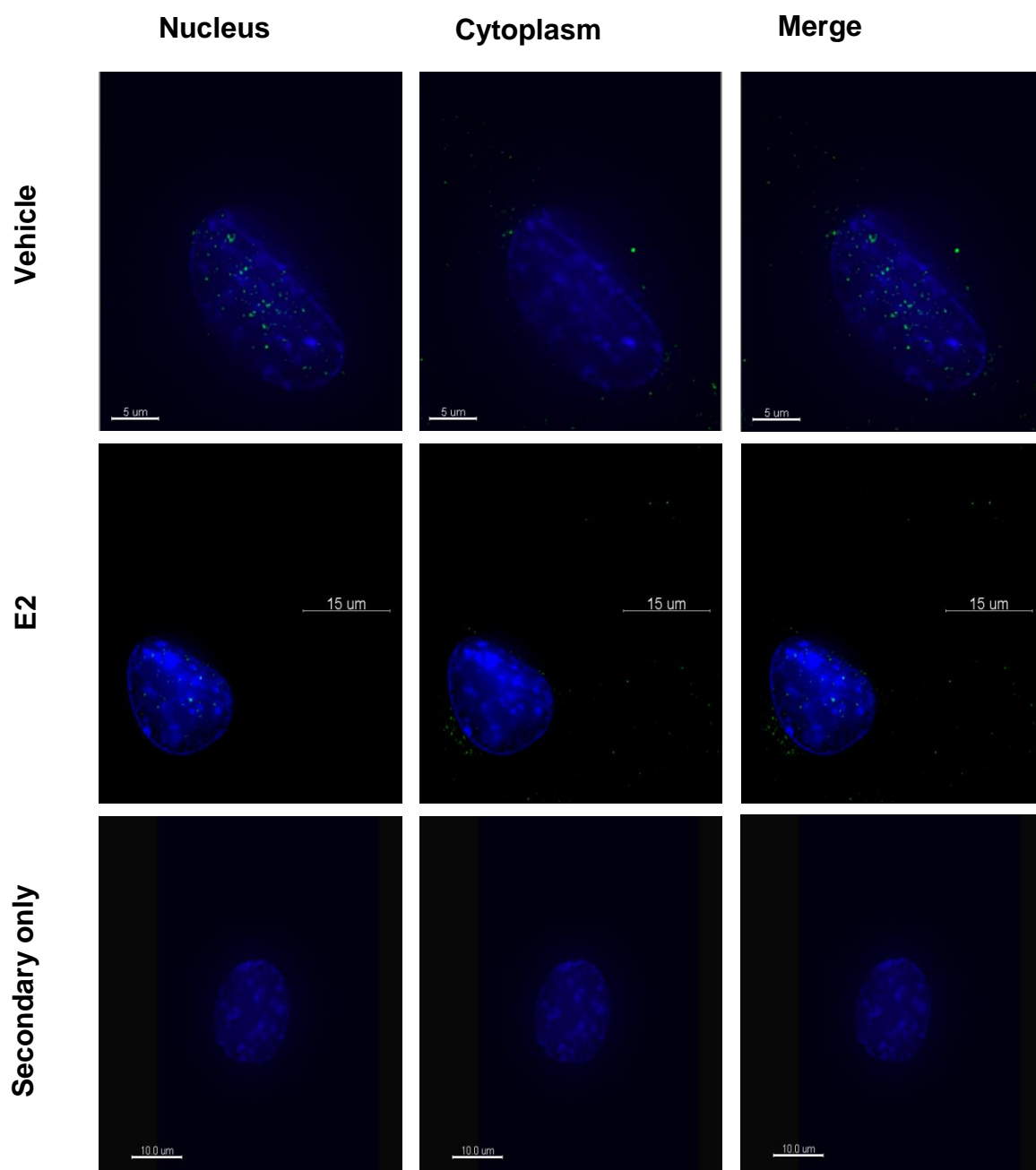


Figure 6. Immunostaining for PNRC2 after E2 treatment. PNRC2 localization is marked by the green (FITC) signal whereas the nucleus is stained in blue (DAPI). The secondary only group was not incubated with any primary antibody.

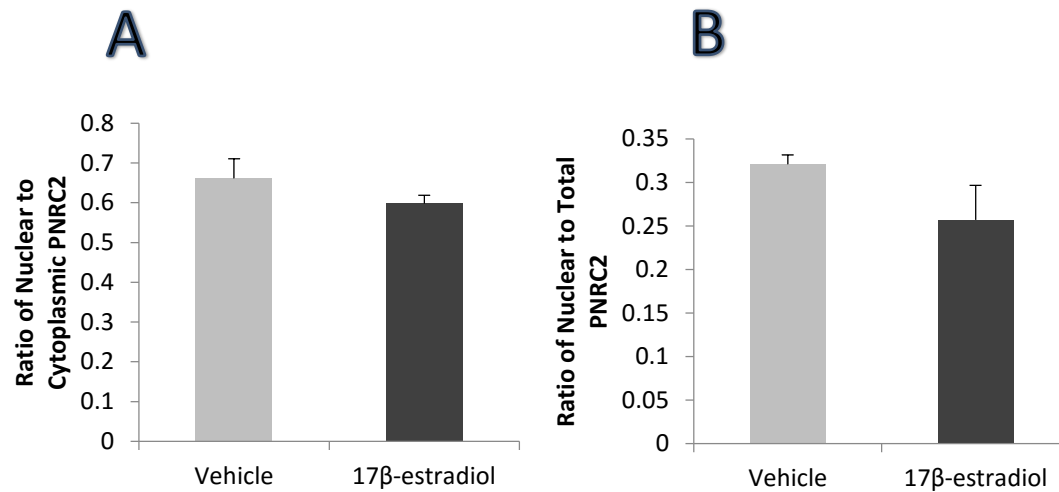


Figure. 7. PNRC2 nuclear to cytoplasmic localization is unchanged with E2 treatment. (A) Ratio of nuclear to cytoplasmic PNRC2 puncta with vehicle or E2 treatment. (B) Ratio of nuclear to total PNRC2 puncta with vehicle or E2 treatment. (N=3) Two sample t-test was performed to test for significance. * indicates $p < .05$

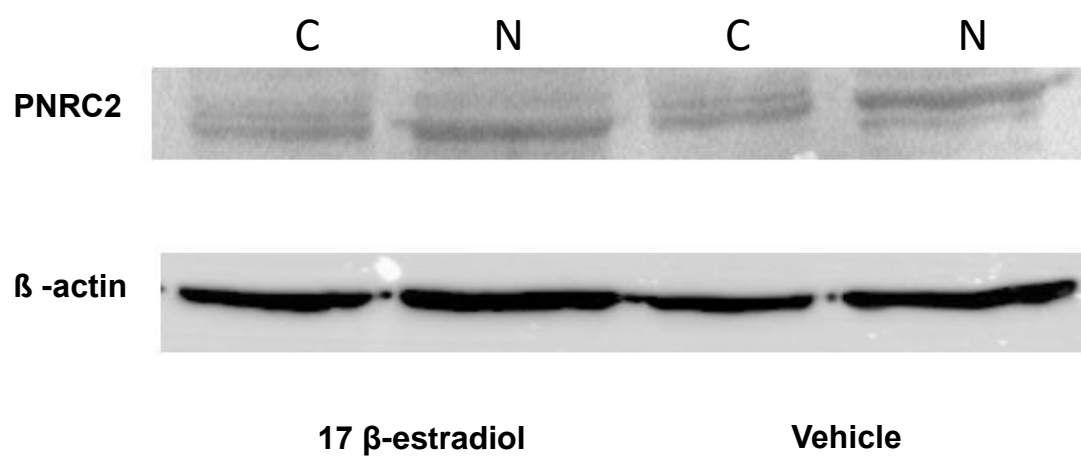


Figure 8. PNRC2 levels after nuclear and cytosolic extraction are unchanged with E2 treatment. β -actin was used to compare nuclear and cytoplasmic PNRC2 levels between E2 and vehicle treated cells.

Next, I tested whether E2 altered the co-localization of PNRC2 with DCP1a. I hypothesized that E2 treatment would decrease the association of PNRC2 and DCP1a, since more PNRC2 would be bound to ERs in the presence of E2. Co-localization analysis was performed using IMARIS. The max intensity of the DCP1a signal was then analyzed for each PNRC2 puncta to evaluate the level of co-localization between these two proteins. It was determined that a DCP1a intensity max of 420 was the threshold in which the red DCP1a signal was reliably seen to overlap with the green PNRC2 signal (Fig.10a). Therefore, co-localization of PNRC2 and DCP1a was defined to be PNRC2 surfaces exhibiting a DCP1a intensity max of over 420. When puncta with these specific criteria were analyzed, it was determined that there was a significant decrease in the co-localization of PNRC2 and DCP1a with E2 treatment (Fig.10b).

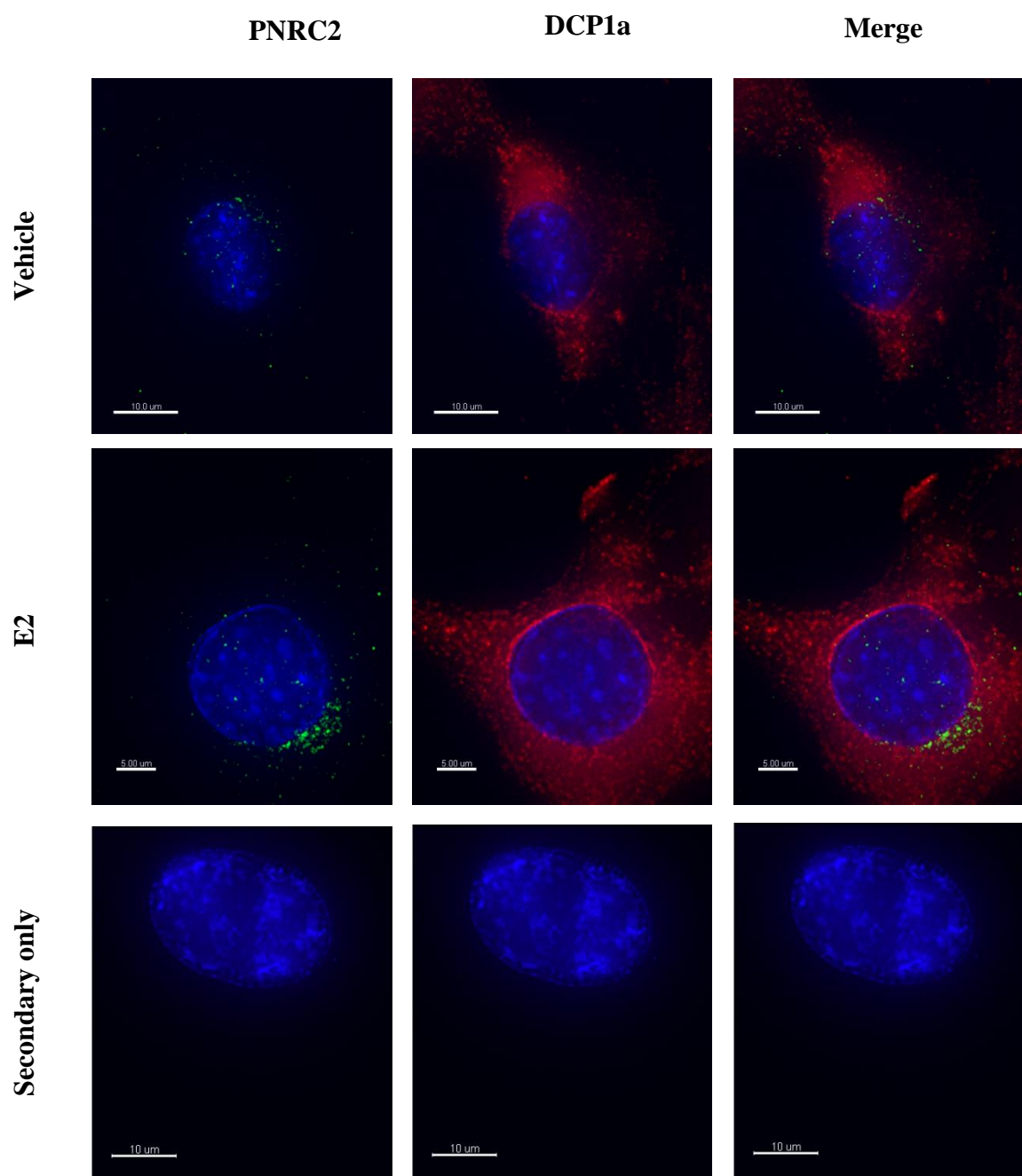


Figure 9. Immunostaining for PNRC2 and DCP1a with E2 treatment. PNRC2 is shown in green (FITC), DCP1a is shown in red (Texas Red), and the nucleus is stained in blue (DAPI). The secondary only group was not incubated with any primary antibody.

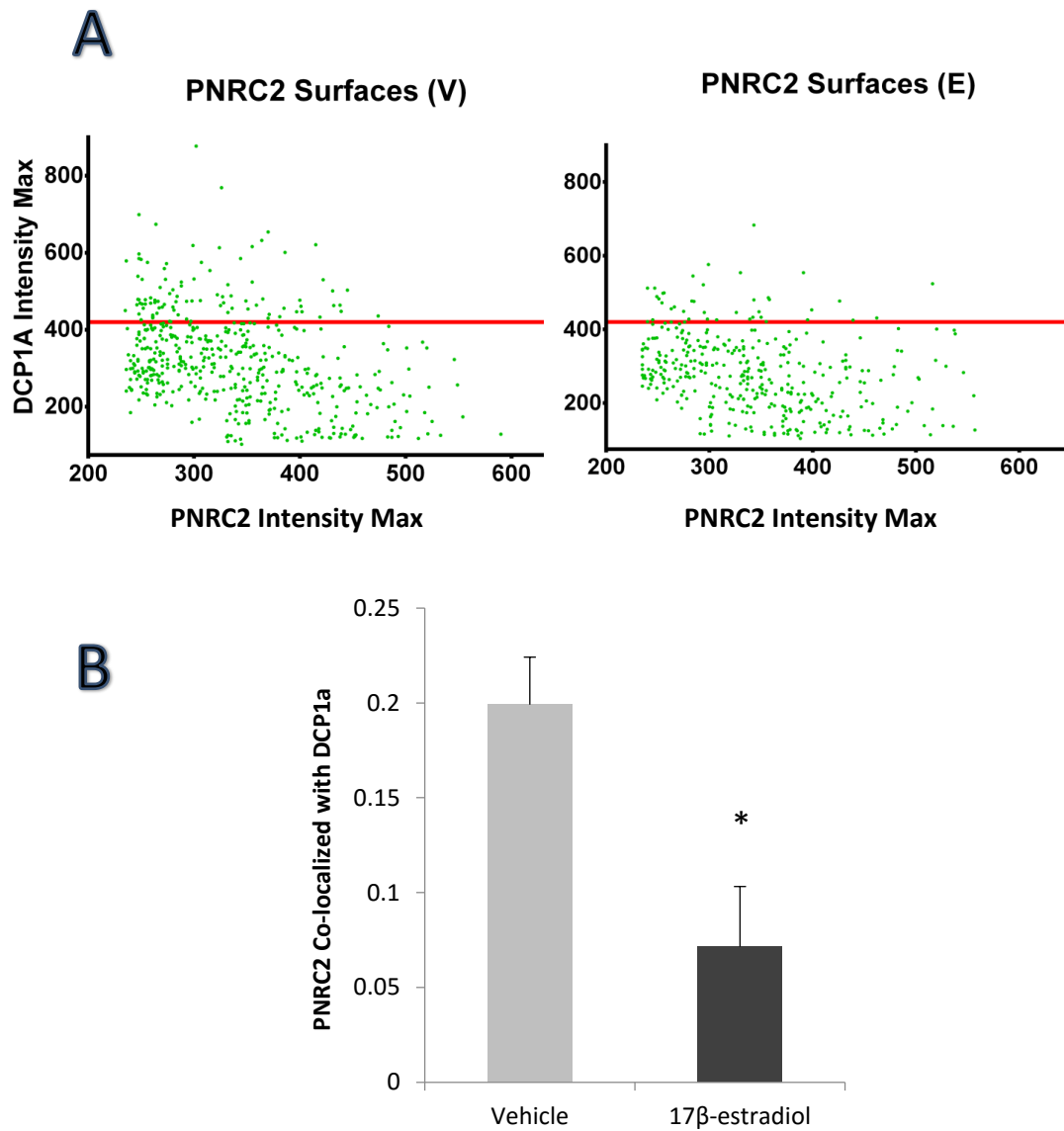


Figure 10. PNRC2 and DCP1a co-localization decreases with E2 treatment.
 (A) Plot of PNRC2 and DCP1a intensity max with vehicle or E2 treatment. (B)
 Percent of PNRC2 co-localized with DCP1a with vehicle or E2 treatment. (N=3)
 Two sample t-test was performed to test for significance. * indicates $p < .05$

In order to ensure that the DRB treatment was successfully inhibiting transcription, I tested the levels of several immediate early genes following 2 hours of DRB treatment. I hypothesized that inhibiting transcription would decrease the abundance of immediate early genes. As expected, RT-qPCR results showed that C-Fos, C-Jun, and C-Myc levels were all decreased following DRB treatment (Fig. 11). C-Fos and C-Myc levels were significantly decreased compared to the control group, suggesting that the DRB treatment was successfully inhibiting transcription (Fig. 11).

I then tested the efficacy of the PNRC2-siRNA to determine if PNRC2 protein abundance would decrease with siRNA transfection. As expected, the IVB cells transfected with scrambled siRNA did not affect the protein levels of PNRC2; however, the PNRC2 siRNA significantly knocked down PNRC2 levels from the non-treated control group (Fig. 12). Densitometry analysis relative to β -actin revealed that there was about a 50% knockdown of PNRC2 protein levels with the PNRC2 siRNA (Fig.12). These data indicate that the PNRC2 siRNA was successfully able to knockdown PNRC2 expression.

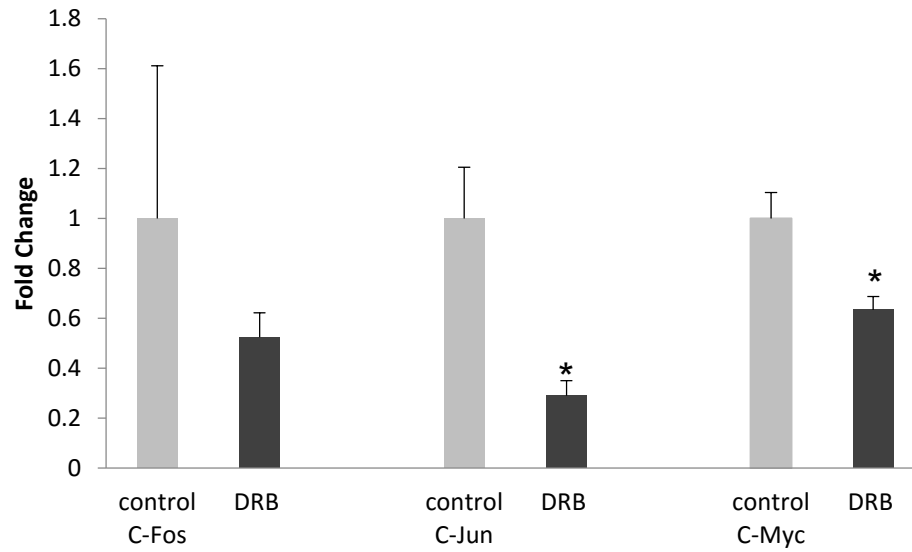


Figure 11. DRB decreases the levels of immediate early genes. RT-qPCR results showing differences in C-Fos, C-Jun, and C-Myc levels with DRB treatment. (N=3) Two sample t-test was performed to test for significance. * indicates $p < .05$

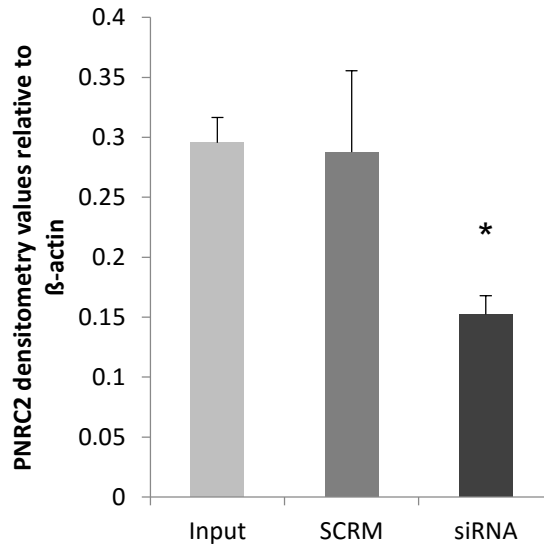
A**B**

Figure 12. PNRC2-siRNA knocks down PNRC2 protein levels. (A) PNRC2 protein levels with no treatment, scrambled siRNA, and PNRC2 siRNA (B) PNRC2 densitometry values relative to β -actin (N=3) Two sample t-test was performed to test for significance. * indicates $p < .05$

Finally, to determine whether PNRC2 has a functional role in miR-181a stabilization, I tested the hypothesis that knocking down PNRC2 protein levels would abolish the E2 mediated stabilization of miR-181a. The results indicated that E2 treatment increased miR-181a levels when transcription was inhibited with DRB (Fig. 13a). However, when PNRC2 expression was knocked down, an increase in miR-181a was no longer observed (Fig.13b). These findings suggest that PNRC2 has an important role in the E2-mediated stabilization of miR-181a.

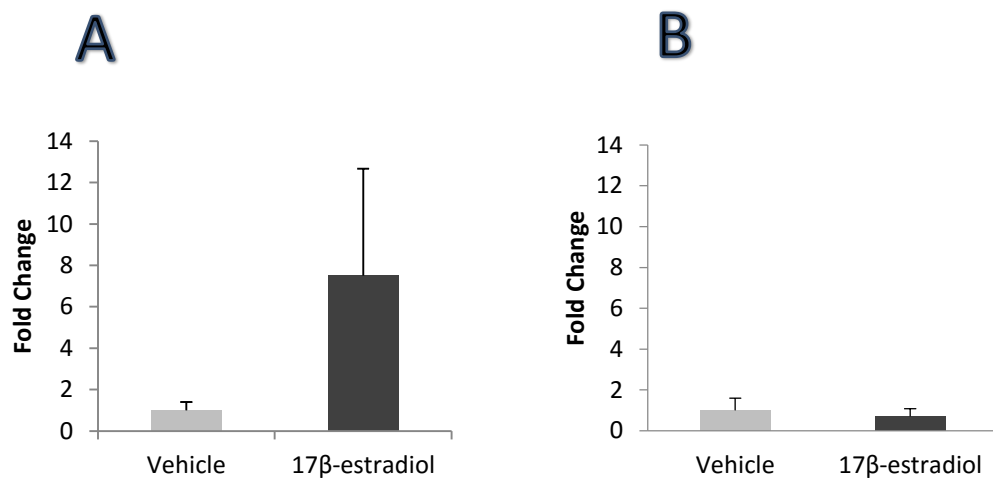


Figure 13. The E2-mediated stabilization of miR-181a is abolished with PNRC2 knockdown. (A) RT-qPCR results showing fold change in miR-181a levels between estrogen-treated and vehicle-treated IVB cells following DRB treatment. (B) RT-qPCR results showing fold change in miR-181a levels between estrogen-treated and vehicle-treated IVB cells following siRNA mediated PNRC2 knockdown and DRB treatment (N=3). Two sample t-test was performed to test for significance with * indicating $p < .05$.

DISCUSSION

Overall, these studies revealed the novel finding that PNRC2 is required for the E2-mediated stabilization of miR-181a. While previous research has examined the role of PNRC2 in mRNA degradation (Lai *et al.*, 2012, *Structure*), there has not been any investigation, to our knowledge, into PNRC2's role in miRNA degradation. Furthermore, the present study found that E2 treatment decreased PNRC2 co-localization to DCP1a, suggesting less downstream activation of the degradation machinery. Lai *et al.* previously found that disrupting this interaction of PNRC2 and DCP1a inhibited mRNA degradation (Lai *et al.*, 2012, *Structure*). While the present study did not directly investigate the involvement of XRN-1, activated DCP1a has been shown to recruit XRN-1 through the scaffolding protein EDC4 (Enhancer of mRNA-decapping protein 4) (Chang *et al.*, 2014, *Nucleic Acids Res.*). Since XRN-1 has also been implicated in miRNA degradation as well as mRNA degradation, it is conceivable that an inhibition in XRN-1 recruitment due to a decrease in PNRC2-DCP1a co-localization leads to miRNA stabilization.

Interestingly, there was no difference in the ratio of nuclear to cytoplasmic PNRC2 with E2 treatment, suggesting that PNRC2 is not shuttled back and forth from the nucleus. Rather, there seems to be consistent levels of PNRC2 in the nucleus and the cytoplasm that remain unchanged with E2 treatment (Fig. 7). Previous immunostaining experiments showed that PNRC2 was primarily localized to the nucleus with only a faint detection in the cytoplasm (Zhou *et al.*, 2000, *Mol. Endo.*). While our data did reveal more PNRC2 puncta in the nucleus

compared to the cytoplasm, more cytoplasmic PNRC2 was observed compared to what was seen by Zhou *et al.* However, Zhou *et al.*, used a different cell line derived from African green monkey kidney tissue in their experiments (Cos-1), and this could explain the differences that were observed.

Since E2 treatment did not decrease PNRC2 levels in the cytoplasm, it is still unknown how E2 treatment decreased PNRC2 co-localization with DCP1a. PNRC2 was not the limiting factor, and PNRC2 function was not altered by its translocation into the nucleus in a E2-dependent manner. This suggests that PNRC2's role as a coactivator for estrogen receptors in the nucleus is independent from its role in the decapping machinery. Therefore, there has to be some other mechanism of E2 action that decreases the co-localization of PNRC2 and DCP1a.

One possible mechanism of E2 decreasing PNRC2 co-localization with DCP1a is through an interaction with the MAPK signaling pathway. E2 has been shown to induce Shc (Src-homology and collagen homology) phosphorylation which promotes the binding of two Grb-2 (growth factor receptor bound protein 2) adaptor proteins (Song *et al.*, 2002, *Mol. Endocrinology*). Interestingly, PNRC2 has also been shown to interact with Grb2 to suppress nuclear receptor-mediated signaling pathways (Zhou *et al.*, 2004, *Oncogene*). Therefore, if Grb2 is recruited to the estrogen receptor in a E2-dependent manner following Shc phosphorylation, this mobilization of Grb2 to Shc could provide binding opportunities for PNRC2. In this scenario, PNRC2 would not be translocated into the nucleus upon treatment with E2; rather, its interaction with Grb2 in the

cytoplasm would prevent PNRC2 binding to DCP1a, and this would consequently suppress XRN1 recruitment and miRNA degradation.

Attempts to co-immunoprecipitate PNRC2 and DCP1a with E2 treatment have been unsuccessful, so the present study could not confirm the interaction between these two proteins. However, PNRC2 and DCP1a interaction has been previously reported using a yeast two-hybrid assay (Lai *et al.*, 2012, *Structure*). Similarly, PNRC2 interaction with nuclear receptors has also been reported using a yeast two-hybrid assay system (Zhou and Chen, 2001, *Nucleic Acids Research*). Other studies have overexpressed PNRC2 and nuclear receptor levels before successfully conducting immunoprecipitation experiments (Cho *et al.*, 2015, *PNAS*). Therefore, it could be the case that the low, endogenous expression of PNRC2 made it difficult to detect in previous Co-IP experiments. Crosslinking of intracellular proteins with DSP (dithiobis succinimidyl propionate) did not improve efforts to immunoprecipitate PNRC2 and DCP1a, suggesting that the protein:protein interactions could be indirect or transient.

Moreover, it is crucial to note that altered PNRC2 signaling alone would not account for the miRNA specific effect of E2 since decreased activation of the degradation machinery would stabilize miRNAs in a global manner. This suggests that PNRC2 signaling is only one aspect of the mechanism of E2-mediated miRNA stabilization. It can be proposed then that the disruption of PNRC2-DCP1a association *and* mRNA target upregulation have an additive effect in the E2-mediated stabilization of miR-181a. It could be the case that transcriptional regulation of mRNA targets by E2 determines the miRNA specific

effect of stabilization or destabilization, while PNRC2 signaling either potentiates or negates the overall effect.

However, as previously described, PNRC2 has diverse roles in intracellular signaling. Not only can it bind and activate DCP1a, it can suppress the MAPK signaling pathway by binding to Grb2 (Zhou *et al.*, 2004, *Oncogene*). Additionally, PNRC2 has been shown to be relatively promiscuous in its ability to bind many different types of nuclear receptors. Recently, PNRC2 was shown to bind the glucocorticoid receptor (GR) to post-transcriptionally regulate mRNA degradation (Cho *et al.*, 2015, *PNAS*). Therefore, knocking down PNRC2 expression could have resulted in unanticipated downstream signaling events. Upon further investigation of PNRC2's role in miRNA stabilization, it would be imperative to consider how differences in the cellular environment could diminish or potentiate PNRC2's role in miRNA stabilization as E2 is likely not the only stimulus that alters its overall effect.

In conclusion, the present findings support the hypothesis that estrogen-mediated stabilization of miR-181a will be abolished upon knockdown of PNRC2. These findings suggest an importance of the PNRC2 signaling pathway in mediating the stabilizing effect of E2 on mature miR-181a levels.

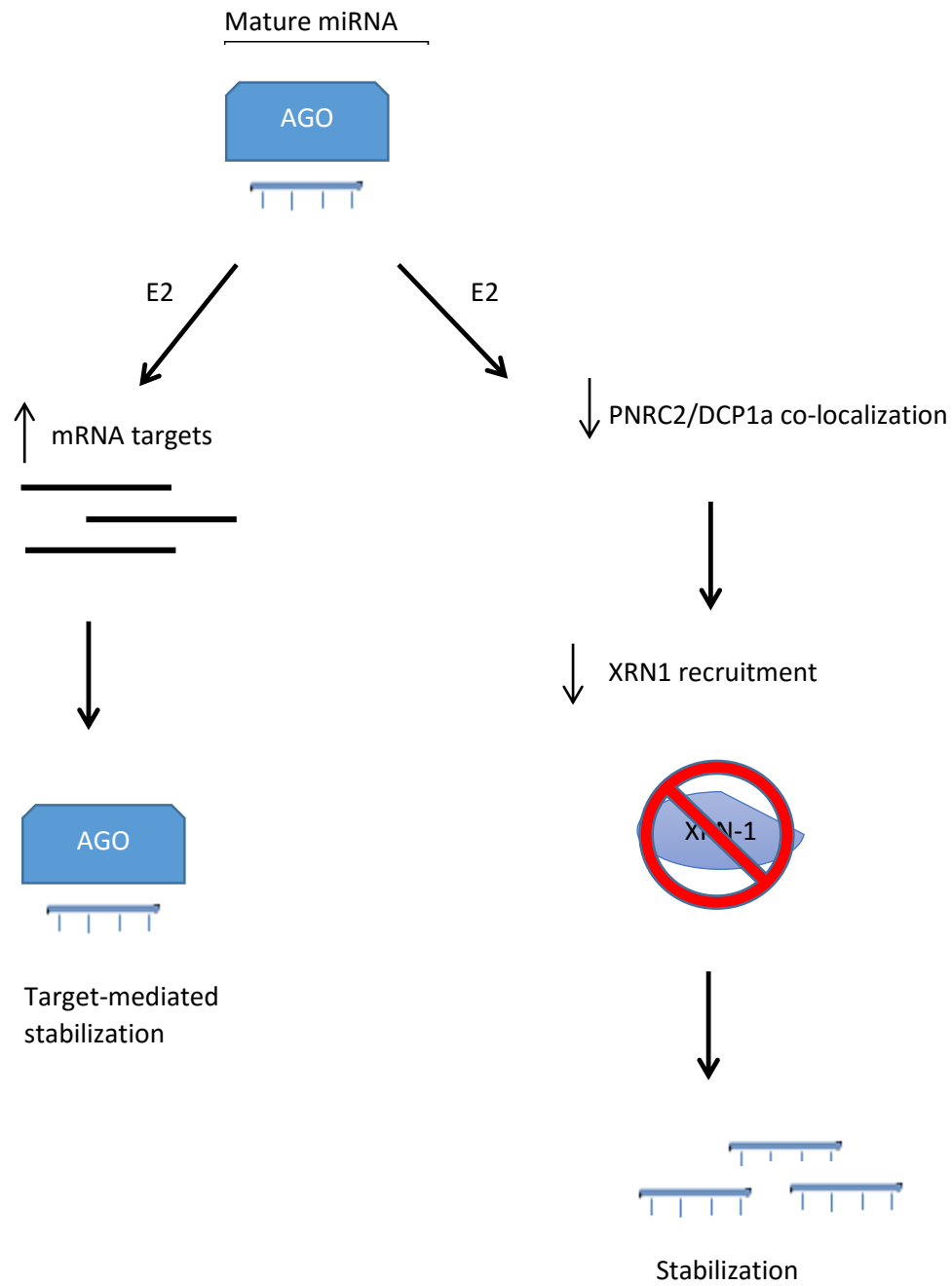


Figure 14. Proposed mechanisms of E2-mediated miRNA stabilization

CHAPTER FIVE

CONCLUSIONS AND FUTURE DIRECTIONS

The primary goal of this project was to determine how E2 altered the expression of specific miRNAs in the rat brain. To that end, it was found that E2 increased miR-181a levels in a rat hypothalamic cell line, and this increase was due to E2 stabilizing the mature miR-181a transcript. Furthermore, the E2-mediated stabilization of miR-181a was found to be dependent on the endogenous pool of RNA as well as PNRC2 expression.

The E2-mediated stabilization of miR-181a could potentially have adverse consequences which lead to disease. For example, it has been previously shown in the literature that E2 can promote metastasis in breast cancer cells (Zheng *et al.*, 2011, *PLOS*). Additionally, dysregulation of the TGF- β signaling pathway has also been studied extensively in breast cancer progression and metastasis as it seems to have a dual role in either suppressing or initiating pro-oncogenic effects (Sato *et al.*, 2014, *Breast Cancer Research*). Interestingly, one of the ways in which TGF- β can promote breast cancer metastasis is to upregulate miR-181a levels (Taylor *et al.*, 2013, *JCI*). Activation of TGF- β receptors leads to the phosphorylation and subsequent activation of R-SMAD 2/3 which can translocate into the nucleus to function as a transcription factor to initiate the transcription of miR-181a. Additionally, the R-SMAD 2/3 dimer can also interact with the Drosha: DGCR complex to promote maturation of

the pri-miRNA to the precursor form, which gives rise to the possibility of the increased efficiency of miR-181a production (Hata and Davis, 2009, *Cytokine and Growth Factor Reviews*). Therefore, one way in which E2 could be promoting metastasis is by allowing for the pro-oncogenic miR-181a to exert its effects for a longer period of time by mirroring, or perhaps exacerbating, the effects of TGF- β mediated miR-181a upregulation.

In the context of the brain, prolonged miR-181a activity due to stabilization could have similarly detrimental effects. miR-181a was found to be enriched in the neuronal synapses of rat primary cortical and hippocampal neurons, and its overexpression decreased GluA2 levels, which is a component of AMPARs. The miR-181a-induced reduction of GluA2 ultimately led to decreased dendritic spine formation (Saba *et al.*, 2011, *Mol. Cell. Biol.*). Therefore, E2-mediated stabilization of miR-181a could negatively regulate synaptic plasticity and neurotransmission in the brain.

One limitation of the present research was that it did not investigate the effect of E2 on miRNA degradation in other tissues. Previous research has indicated that E2 can initiate tissue-specific signaling cascades with markedly different cellular responses (Cui *et al.*, 2013, *Trends in Molecular Medicine*). Therefore, the E2-mediation stabilization of miR-181a that was observed in a rat hypothalamic cell line might not be present in cell lines derived from other tissues. Similarly, *in vitro* studies using IVB cells do not consider the role of glial cells such as astrocytes and microglia in mediating the effects of E2. Therefore, further research with various tissue types is required to determine if the observed

effect of stabilization is specific to the brain and if this effect is also seen in an *in vivo* system.

Another limitation of the present research was that it was not able to distinguish which class of RNAs was important in the E2-mediated stabilization of miR-181a. While target-mediated stabilization by mRNA upregulation seems to be a likely mechanism of stabilization, follow-up miRNA degradation experiments co-incubating radiolabeled ³²P-miR-181a and a custom oligonucleotide sequence complementary to the seed sequence would provide a more detailed understanding of the mechanism of miR-181a stabilization.

Furthermore, it would be interesting to determine if miR-181a localization *in vitro* is altered after E2 treatment. RISC, or RNA induced silencing complex, is generally thought to shield miRNAs from nucleases present in the cytoplasm. Therefore, when loaded onto a complex of proteins, miRNAs are thought to be more stable. Interestingly, key components of RISC, such as the Argonaute protein family, have been reported to localize in p-bodies and other RNPs (Sen *et al.*, 2005, *Nat Cell Bio*). It has also been suggested that miRNA-induced translational repression is achieved upon the delivery of mRNAs to these p-bodies (Liu *et al.*, 2005, *Nat Cell Bio*). Therefore, if miRNAs are localized to p-bodies after E2 treatment, this could be one mode of stabilization. This would represent a novel mechanism of miRNA stability that would not necessarily include a miRNA-to-mRNA interaction or some other enzymatic process. Such a finding would be of importance, because it would show that miRNA stability can be affected simply by changing its localization to different foci within the cell.

In conclusion, E2 was found to alter the expression of specific miRNAs—namely miR-181a—by stabilizing the mature transcript in a manner that is dependent on both PNRC2 and endogenous RNA. The present findings add another layer of complexity in the study of miRNA and the development of miRNA-specific drugs. With the knowledge that hormonal triggers can regulate miRNA turnover, this research necessitates the development of miRNA-targeted drugs that are sex specific. Furthermore, the elucidation of miRNA stability is important for proper understanding of the homeostatic brain as well as the mechanisms by which miRNA can be involved in pathological conditions. The research presented here fills important gaps in our understanding of miRNA biology and provides an underlying foundation for future research into hormonal alterations of miRNA stability.

CHAPTER SIX

GENERAL METHODS

***in vitro* Preparations**

IVB cells, a neuronal cell line derived from rat hypothalamus, were grown to 70% confluency in Dulbecco's Modified Eagle Medium (Corning) supplemented with 10% Fetal Bovine Serum. (IVB cells were generously provided by Dr. John Kaskow at the University of Cincinnati, OH). After 48 hours of incubation in media with charcoal/dextran treated FBS, cells were treated with 100 nM E2 or an equivalent volume of ethanol (vehicle) for 2 hours. Cells were immediately lysed using a mild, Nonidet-P40 (NP40) buffer to preserve protein: protein interactions. The lysate was then collected and protein concentration was quantified using a BCA assay (ThermoFischer Scientific). All cells were between passage 19 and 23.

miRNA Degradation Assay

In order to observe the degradation kinetics of miR-181a and miR-124, synthetic oligonucleotide constructs were created (Integrated DNA Technologies) with the exact sequence of the mature strand (miR181a: AACAUUCAACGCUGUCGGUGAGU; miR-124: UAAGGCACGCGGUGAAUGCC). These single stranded oligonucleotide sequences were then radiolabeled on the 5' end using [γ 32P] ATP (3000 Ci/mmmol). 10 fmols of the newly radiolabeled sequence were then incubated

with 20 µg of protein from the lysate prepared above. Incubation of radiolabeled constructs with the lysate was stopped at 5 different time points with formamide loading buffer: T0, 15 minutes, 1 hour, 2 hours, and 4 hours. The resulting mixture was then separated by electrophoresis on an 8% urea gel. Finally, the gel was visualized by phosphorimaging to detect levels of the radiolabeled miRNA at the various time points.

Micrococcal Nuclease Treatment

1 µl of MNase with a 2×10^6 gel units/ml concentration was added to 100 µg of lysate. The reaction was stopped after 30 minutes by adding excess EGTA to a final concentration of 10mM. After MNase treatment, the miRNA degradation assay was performed as described above.

Immunostaining for PNRC2 and DCP1a

IVB cells, grown on glass coverslips, were treated with E2 or vehicle treatment as described above. Upon 70% confluency, the cells were fixed by incubating with 100% methanol for 5 minutes. Following fixation, 0.25% Triton X-100 was applied to permeabilize the samples. The cells were then incubated with 1% BSA in PBST (PBS + 0.1% Tween 20) for 30 minutes to prevent nonspecific binding. Next, the sample was simultaneously incubated with a PNRC2 antibody raised in goat (Santa Cruz) and a DCP1a antibody raised in rabbit (Sigma) overnight at 4°C. After washing with PBS, the cells were then incubated with the mixture of an anti-rabbit secondary antibody conjugated to Texas Red and an anti-goat secondary antibody conjugated to FITC for 30 minutes at room temperature. The glass coverslips containing the IVB cells were mounted on a

slide to be visualized by deconvolution microscopy. 15 representative pictures were taken from each treatment group, and the images were analyzed using IMARIS software to determine subcellular localization.

IMARIS Analysis

In order to analyze PNRC2 puncta localization relative to the nucleus, a masked surface was created around the DAPI signal using IMARIS. This mask was then used to create two separate channels displaying PNRC2 puncta within the masked surface and outside the masked surface. The number of PNRC2 puncta in each channel was then determined by applying an algorithm based upon fluorescence intensity, sphericity, and volume.

Co-localization analysis was performed by analyzing the fluorescence intensity of both the PNRC2 and DCP1a surfaces. A threshold value of 420 was set for the DCP1a fluorescence intensity as this was the minimum limit in which the red (Texas Red) signal began to overlap with the green (FITC) PNRC2 signal.

DRB Treatment

IVB cells were treated with 5,6-dichlorobenzimidazole 1- β -D-ribofuranoside (DRB) at a final concentration of 65 μ M for 2 hours in order to inhibit transcription. DRB was diluted in dimethyl sulfoxide at a stock concentration of 65 mM prior to addition to cell culture media.

PNRC2 siRNA Transfection

PNRC2-siRNA (OriGene) was transfected into IVB cells at 70% confluency to a final concentration of 10 nM. Transfection was achieved using a

3:1 ratio of Continuum transfection reagent (Gemini) with the siRNA for 48 hours. Scrambled siRNA (OriGene) was used as a control in all experiments to determine whether there was any nonspecific knockdown of PNRC2 from the duplex due to transfection alone.

Real-Time Quantitative PCR

Total RNA was isolated from samples using TRIzol reagent (Thermo). 2.5 µg of RNA was then used for poly-A tailing and cDNA synthesis (NCode). RT-qPCR was performed using a miR-181a primer along with a 5sRNA primer as a reference gene (IDT).

Statistics

Two-way ANOVA was performed to determine the interaction of time and treatment on miRNA expression in the miRNA degradation assays. Two sample t-tests were performed for all other experiments to determine differences between treated and untreated groups. A p-value of less than 0.05 was considered statistically significant.

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